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Transfrmed micro rganisms with improved pr perties.

Field of the invention

5   The present invention relates to genetic engineering of production microorganisms used
in biotechnology to improve their properties so that they produce useful products more
efficiently. In particular the invention relates to increasing the productivities of
biotechnological processes and the yields of their products such as ethanol or amino
acids from carbon and nitrogen sources such as biomass comprising hexoses, pentoses
10   or their polymers.

Background of the invention

The efficiency of many biotechnological processes is limited by the need of production
15   organisms to balance their metabolic redox reactions. In particular, for each of the
pyridine nucleotide couples (NAD/NADH and NADP/NADPH) the total rate of
oxidation must be equal to the total rate of reduction: otherwise, the couple will be
completely converted into one form (e.g. all in the NAD form or all in the NADH
form), and reactions requiring the other form will become infinitely slow, causing the
20   whole metabolic network of reactions be distorted in an undesirable way (i.e., no longer
provide the desired product).

For example, although the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces
pombe* are very efficient at converting hexoses into ethanol and have many advantages
25   for this process (such as tolerance of high ethanol concentrations and other stresses)
they are unable to ferment xylose to ethanol. Xylose is a major component of plants,
and the inability to convert it to ethanol decreases the efficiency with which renewable
biomass, such as agricultural wastes, can be utilized. However, these yeasts can utilize
xylulose. Some yeasts (e.g. *Pichia stipitis*) can convert xylose to ethanol, although not
30   very efficiently, and contain the enzymes xylose reductase (XR) and xylitol
dehydrogenase (XDH). These enzymes catalyse the sequential reduction of xylose to
xylitol and oxidation of xylitol to xylulose. Transformed *S. cerevisiae* strains have been
constructed containing heterologous XR and XDH, which so possess a pathway to

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convert xylose into the fermentable xylulose (Kötter and Ciriacy [1993]; Tantirungkij *et al.* [1994]; Walfridsson *et al.* [1995]). Although these strains could use xylose for growth and xylitol formation, they did not produce much ethanol. All known XDH enzymes are specific for NAD, whereas all known XR enzymes are either specific for

5   NADPH or have a preference for NADPH. It is believed (Bruinenberg *et al.* [1983]; Bruinenberg [1986]) that conversion of xylose to xylulose by this pathway therefore results in the cellular pool of NADPH being converted to NADP and that of NAD being converted to NADH, after which further metabolism of xylose is greatly hindered. The NADH can be reoxidised under aerobic conditions, but this demands critical control of

10 oxygen levels to maintain fermentative metabolism and ethanol production. In contrast, bacteria that ferment xylose to ethanol efficiently contain xylose isomerase and convert xylose directly into xylulose without oxidation–reduction reactions. Attempts to create yeast that can efficiently convert xylose to ethanol have focused on finding or engineering XR or XDH enzymes with altered coenzyme specificity (Metzger and

15 Hollenberg [1995]) or on expressing xylose isomerase gene in yeast. However, all reported attempts (see, e.g., Amore *et al.* [1989]; Ho *et al.* [1983]; Sarthy *et al.* [1987]; Walfridsson *et al.* [1996]) to construct good xylose–fermenting strains by expressing bacterial xylose isomerase genes in yeasts have failed.

20 As a second example, a major biotechnological process is the fermentation of hexose sugars to ethanol by yeast. The glycolytic pathway from glucose to ethanol is redox neutral, i.e. the amount of NAD reduced in the formation of a certain amount of pyruvate from glucose is exactly the same as the amount of NADH oxidised in the formation of ethanol from the same amount of pyruvate, and NADP(H) is not directly

25 involved in the process. However, yeast growth is not a redox neutral process; the formation of 100 g dry yeast matter from glucose and ammonia is accompanied by the net production of 1.3 moles of NADH and 0.9 moles of NADP (Oura [1972]). This excess NADH is produced mainly by energy yielding catabolism, whereas the excess NADP is produced mainly by biosynthetic pathways (see Oura [1972]). Like other

30 organisms, yeast has distinct pyridine nucleotide systems (NAD(H) and NADP(H)) that have evolved to facilitate these two aspects of metabolism. The excess NADH produced by fermenting yeast is reoxidised to NAD mainly by glycerol–3–phosphate dehydrogenase, resulting in the production of glycerol. In distillery fermentations this

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represents a wasteful diversion of 3–5 % of the carbon source (Oura [1977]). Attempts to decrease the proportion of glycerol to ethanol produced during fermentations have met with little or no success. For example, Björkqvist et al. (1997) deleted each and both of the genes encoding glycerol-3-phosphate dehydrogenase. However, yeasts

5 lacking this enzyme were not only unable to grow under anaerobic conditions, but they also stopped making ethanol.

A third example is the biotechnological production of amino acids. Amino acids have extensive applications in the food, animal feed, medical and chemical industries.

10 Fermentation processes have been developed to produce most amino acids occuring in proteins. The metabolic routes to amino acids first convert a carbon source such as glucose into intermediates such as 3-phosphoglycerate, pyruvate, oxaloacetate or 2-oxoglutarate that are more oxidised than glucose. Their formation produces NADH. Most amino acids are more reduced than the intermediates, but the reactions leading to

15 them from the intermediates almost invariably produce NADP. Apart from the histidine pathway, which is a net NADPH producer, and the pathways to glutamine, glutamate, tyrosine and phenylalanine, which neither consume nor produce NADPH, biosyntheses of all the other 15 amino acids from glucose produce between 1 and 8 moles of NADP per mole of amino acid and simultaneously produce NADH (Neidhardt et al. [1990]).

20 Other reactions are then required to oxidise the NADH and reduce the NADP in order to achieve metabolic balance. This becomes a major factor with production organisms such as Corynebacteria modified and/or selected to produce huge amounts of amino acids on a commercial scale. To dispose of excess NADH, amino acid fermentations are operated under aerobic conditions, and oxygen is consumed in large amounts. To ensure

25 maximum product formation, it is essential continuously to supply adequate amounts of oxygen, typically in the form of oxygen-enriched air (Hirose [1986]). Oxygen deficiencies, e.g., in high cell density fermentations or in cases where oxygen supplementation is uneconomical, typically result in lower product yields and productivities, as part of the carbon source is converted to compounds such as succinate, lactate

30 or both to get rid of excess NADH.

Other examples include the enhanced biosynthesis of nucleotides, lipids and secondary metabolites by modified microorganisms selected or engineered to produce these

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compounds on the industrial scale. During these processes the microorganisms generally produce NADH and a central metabolic intermediate (such as pyruvate) that is more oxidised than the carbon source and reduce this intermediate to the desired product using NADPH. Once again, the microorganisms need to oxidise the excess NADH and

5 reduce the excess NADP, and the yields on carbon source are decreased by the additional metabolic transformations of the carbon source required to achieve redox balance.

In all these examples, excess NADH is reoxidised either by respiration, requiring
10 efficient aeration, or by the formation of unwanted side products, such as glycerol. Aeration on large industrial scales is expensive, and difficult to control exactly. In some processes, such as the fermentation of xylose to ethanol, reduction of excess NADP causes also problems. Important biochemical reactions regenerating NADPH are the oxidative branch of the pentose phosphate pathway (PPP), i.e. the successive reactions
15 of glucose–6–phosphate dehydrogenase and 6–phosphogluconate dehydrogenase, and NADP–linked isocitrate dehydrogenase. Both reactions produce CO_2. In industrial scale operations, this represents both a direct loss of carbon source and an environmental pollution. Furthermore, CO_2 also acidifies culture media, necessitating the use of larger amounts of neutralising agents to control fermentation pH, and has a significant impact
20 on cell physiology in general and amino acid production in particular. For example, CO_2 inhibits enzymes in methionine and purine biosynthesis and has been reported to inhibit product formation in several fermentation processes including production of isoleucine, inosine, fumarate, penicillin and other antibiotics and yeast biomass (Hirose [1986]).

25 A general method to alleviate these problems without using aeration, which is expensive and difficult to control at optimal levels, would be very beneficial. Potential benefits include increased yields on carbon source, decreased energy consumption, significant decreases in CO_2 production and increased specific productivity, which is particularly important in processes using immobilised microorganisms.

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In the major routes of carbon and nitrogen metabolism it is a general rule that most catabolic pathways use the NAD/NADH coenzyme couple in the oxidation–reduction steps, whereas anabolic, synthetic reactions more frequently use the NADP/NADPH

couple. Although the redox potentials (E'o) of these two couples are both close to −0.32 (Kaplan [1960]), the ratios of the reduced and oxidised forms of the two couples are maintained at very different levels in living cells. For example, in aerobic *S. cerevisiae*, NADH/NAD = 0.9 and NADPH/NADP = 3.2 (Sáez and Lagunas [1976]).

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Most pyridine nucleotide dehydrogenases have a marked, often nearly absolute, specificity for one or the other pyridine nucleotide. Some dehydrogenases with the same substrate occur as both NAD− and NADP−specific enzymes. Usually only one of the enzymes is present under certain conditions, or the enzymes are expressed in different

10   cell compartments. Good examples are glutamate dehydrogenases which are subject to complicated control mechanisms usually resulting in only one of the enzymes being dominant under any growth condition (e.g. Courchesne and Magasanik [1988]; Coschigano *et al.* [1991]; Miller and Magasanik [1991]; ter Schure *et al.* [1995]; Dang *et al.* [1996]; Avendano *et al.* [1997]). *S. cerevisiae* contains NAD− and NADP−linked

15   isocitrate dehydrogenases: the cytosol (which is thought of as a single compartment) contains only NADP−linked enzyme and there is another NADP−linked enzyme in the peroxisomes whereas mitochondria (where the matrix, cristae and intermembrane space form three sub−compartments) contains both NAD− and NADP−linked enzymes (Minard *et al.* [1998]). Cells are therefore able to maintain NADH/NAD ratios much

20   lower than the NADPH/NADP ratios, because reactions that transfer reducing equivalents between the two systems (and so would tend to equilibrate them) are restricted. Some bacterial and animal cells contain NAD(P) transhydrogenases (EC 1.6.1.1. and 1.6.1.2). Transhydrogenases are often membrane−bound enzymes with several subunits which are linked to energy production rather than to equilibration of the

25   pyridine nucleotide systems. For the purposes of this patent application, the term "dehydrogenases" does not include the transhydrogenases EC 1.6.1.1 and 1.6.1.2. Many production organisms used in biotechnology, such as *S. cerevisiae* and Corynebacteria do not contain NAD(P) transhydrogenases, and so they appear to be unable to convert NADH *plus* NADP directly into NAD *plus* NADPH and *vice versa*.

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The existence of two pyridine nucleotide systems and the absence of unregulated processes that would equilibrate them, suggests that the efficient growth and reproduction of presently evolved living organisms requires two distinct systems. The reason

may be that a high NADPH/NADP ratio is required to drive biosynthetic reactions, whereas a lower NADH/NAD ratio is better suited for the generation of energy by pathways such as glycolysis and the tricarboxylic acid cycle (Metzler [1977]).

5   Boles *et al.* (1993) studied a mutant *S. cerevisiae* that lacked phosphoglucoisomerase, the enzyme that interconverts glucose-6-phosphate (Glc6P) and fructose-6-phosphate (Fru6P). This strain (a *pgi1*-deletion mutant) is unable to grow on any hexose or pentose, though it can grow on certain mixtures of fructose and glucose (e.g. 2 % fructose plus 0.1 % glucose). The authors found that transformation of the mutant with

10   a genomic library prepared from the mutant itself resulted in certain transformants that were able to grow on glucose alone, although 3- to 4-times slower than wild type, and contained plasmids comprising the *GDH2* gene. This gene encodes an NAD-linked glutamate dehydrogenase. The authors argued that the simultaneous presence of substantial activities of both NADP- and NAD-linked glutamate dehydrogenases

15   enabled the *pgi1*-deletion mutant to grow on glucose by metabolising it through the PPP and converting the resulting NADPH into NADH, which could then be re-oxidised by functional mitochondria. Thus, these mutants were proposed to convert NAD plus NADPH into NADH plus NADP, which is the opposite transformation to that required of industrial production microorganisms (see above). Furthermore, their ability to

20   survive on glucose was strictly dependent on the presence of functional mitochondria and oxygen and they were unable to ferment sugars into ethanol (Boles *et al.* [1993]).

Summary of the invention

25   According to the present invention a microorganism such as a fungus or a bacterium is transformed with at least one recombinant DNA molecule encoding or otherwise causing the expression of at least one enzyme that causes the functional coupling of the oxidation and reduction of substrates by two pyridine nucleotide-linked dehydrogenase reactions with different specificities for the NAD/NADH and NADP/NADPH coenzyme

30   couples and so facilitates the transfer of electrons between the two coenzyme couples through the said substrates. The enzyme or enzymes can thus be one or more members of a pair of pyridine nucleotide-linked dehydrogenases that have at least one common substrate but different pyridine nucleotide specificities. Biotechnological processes in

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which a net oxidation of one pyridine nucleotide coenzyme couple occurs together with a net reduction of the other are carried out more efficiently by the transformed microorganism according to the invention than by the corresponding non-transformed microorganism, and the aeration of such processes can be decreased and made more

5   flexible. These processes include the fermentation of carbohydrate to ethanol by growing microorganisms, the fermentation of xylose to useful products and the commercial production of amino acids, nucleotides, lipids and secondary metabolites by microorganisms.

10   Preferable microorganisms for the purposes of this invention are yeasts, filamentous fungi and bacteria. Preferable yeasts belong to the genus *Saccharomyces*, and are especially strains of the species *Saccharomyces cerevisiae*; the genus *Schizosaccharomyces*, and are especially strains of the species *Schizosaccharomyces pombe*; and the genus *Pichia*, and are especially strains of the species *Pichia stipitis*, as well as *Candida*

15   spp. or *Pachysolen* spp. Useful filamentous fungi include e.g. *Trichoderma*, *Aspergillus*, *Neurospora*, *Fusarium*, *Paecilomyces* and *Penicillium*. Particularly suitable bacterial genera include Corynebacteria, especially the strains *Corynebacterium glutamicum*, as well as Brevibacteria, such as *Brevibacterium flavum* and *B. lactofermentum*.

20   **Brief description of the drawings**

Figure 1. The genetic map of pAOS66 with the relevant genes, expression cassettes and restriction sites indicated.

25   **Figure 2.** Alignment of the amino acid sequence deduced from a partial sequence of the gene encoding the malic enzyme of *Aspergillus nidulans* with some known malic (SEQ ID NO:1) enzymes. The database numbers (SwissProt) for the known malic enzymes are: P23368 (SEQ ID NO:3) (SEQ ID NO:4) (human malic enzyme), P40375 (*S. pombe* malic enzyme), P36013 (*S. cerevisiae* malic (SEQ ID NO:5) enzyme). Partial sequence of the gene encoding the malic enzyme of *Trichoderma* (SEQ ID N:2)

30   *reesei* was obtained at VTT, Biotechnology and Food Research. Amino acids identical at least in three of the sequences are shadowed in grey.

Figure 3. Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain

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expressing *GDH2* (H1803, solid symbols) and control strain (H1805, open symbols): comparison of growth and xylose utilization rates.

Figure 4. Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain
5   expressing *GDH2* (H1803, solid symbols) and control strain (H1805, open symbols): comparison of ethanol and xylitol production rates.

Figure 5. Xylose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1803, solid line) and control strain (H1805, dotted line): compari-
10   son of carbon dioxide evolution rates.

Figure 6. Specific enzymatic activities of NADP-glutamate dehydrogenase (NADP-GDH) and NAD-glutamate dehydrogenase (NAD-GDH) for strains H1805 (control) and H1803 at time points of 26 and 96 hours.

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Figure 7. Glucose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *GDH2* (H1791, solid line) and control strain (H1793, dotted line): comparison of carbon dioxide evolution rates.

20   **Figure 8.** Biomass profiles for xylose batch fermentations of *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2195) and control strain (H2191).

Figure 9. Overall specific metabolic rates (C-mmol/g-cell h) from batch fermentations of *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2195) and control
25   strain (H2191).

Figure 10. Time profiles for ethanol production from xylose in batch fermentations of *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2222) and control strain (H2221). Ethanol concentrations are normalized with corresponding biomass
30   levels.

Figure 11. Overall specific metabolic rates (C-mmol/g-cell h) from batch fermentations of *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2222) and control

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strain (H2221).

Figure 12. Biomass time profiles for xylose batch fermentations of *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2222) and control strain (H2221).

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Figure 13. Biomass profiles for xylose batch fermentations of *Schizosaccharomyces pombe* recombinant strain H2369 expressing malic enzyme, and the control strain H2370.

10 **Figure 14.** Time profiles for volumetric ethanol production from xylose in *Schizosaccharomyces pombe* recombinant strain H2369 expressing malic enzyme, and the control strain H2370.

**Figure 15.** Separation of *BamHI* digestion products of the vectors from the
15 *Corynebacterium* transformants described in Example 21. **Lane 1:** digestion of the vector from ATCC 21253 transformant, **Lane 2:** Not relevant for this experiment, **Lane 3:** digestion of the vector from ATCC 21799 transformant (VTT E-992103).

20 **Detailed description of the invention**

The central teaching of the present invention is a method to enhance biotechnological processes by transforming production microorganisms with genes for enzymes that tend to equilibrate the two pyridine nucleotide systems that coexist in living cells. Surpris-
25 ingly, although cells have evolved two distinct pyridine nucleotide systems, which are maintained at distinct redox potentials, and such equilibration reactions are apparently prohibited in naturally evolved cells, it is now disclosed that these reactions promote the metabolic pathways desired for product formation by the engineered or selected microorganisms used in many biotechnological processes, and thereby benefit those
30 processes.

In its first embodiment the present invention provides a microorganism which is transformed with at least one recombinant DNA molecule encoding or otherwise causing the

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expression of at least one of a pair of dehydrogenases with opposite coenzyme specific-
ities for NAD/NADH and NADP/NADPH but at least one common substrate (S in
equations (1) and (2)) in such a way that both members of the pair are simultaneously
expressed in the same subcellular compartment, preferably the cytosol. This results in a

5   functional coupling of the dehydrogenases catalysing reactions (1) and (2). It is not a
necessary part of the invention, but neither is it excluded, that the two dehydrogenases
should physically associate within the transformed cell. The functional coupling allows
the following reactions to occur, which tend to equilibrate the NAD/NADH and
NADP/NADPH coenzyme couples:

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(1) $NADP + SH_2 \iff S + NADPH$

(2) $S + NADH \iff SH_2 + NAD$
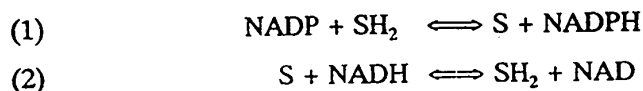
Simultaneous operation of reactions (1) and (2) might be expected to proceed until the

15   NAD/NADH and NADP/NADPH ratios are almost identical, because the redox
potentials of the two couples are very similar. However, the inventors show here that
when production microorganisms are transformed in this way, the efficiency with which
raw material is converted into useful products and the yields of products on biomass are
substantially increased.

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It is to be noted that the tendency to equilibrate the two pyridine nucleotide couples
brought about by reactions (1) and (2) (and also by reactions (3) to (5), (6) to (8) and
(11) to (12) below) is caused by transfer of electrons through the substrates of pyridine
nucleotide-linked dehydrogenases (SH_2 in reactions (1) and (2) and reactions (3) to (5),

25   malate in reactions (6) to (8) and glutamate in reactions (11) and (12)). This
distinguishes the present invention from systems in which electrons are transferred from
NAD(P)H to NAD(P) by so-called transhydrogenases (e.g., EC 1.6.1.1 and 1.6.1.2).
Kojima *et al.* (1996: EP 0 733 712 A1) have described a system in which
transhydrogenase may be utilised in certain bacteria as a means of converting NADH

30   generated through the TCA cycle into NADPH.

Several pairs of dehydrogenases are known which share common substrates but have
different pyridine nucleotide specificities. For example, there are both NAD- and

NADP-linked forms of glutamate dehydrogenase (EC 1.4.1.2 and 1.4.1.4), isocitrate

dehydrogenase (EC 1.1.1.41 and 1.1.1.42), aldehyde dehydrogenase (EC 1.2.1.3 and

1.2.1.4), alcohol dehydrogenase (EC 1.1.1.1 and 1.1.1.2), malate dehydrogenase (EC

1.1.1.37 and 1.1.1.82), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8 and 1.1.1.94),

5   xylose-1 dehydrogenase (EC 1.1.1.175 and 1.1.1.179), glyceraldehyde-3-phosphate

dehydrogenase (EC 1.2.1.12 and 1.2.1.13), orotate reductase (EC 1.3.1.14 and 1.3.1.15)

and ferredoxin reductase (EC1.18.1.2 and 1.18.1.3) but any appropriate pair of dehydro-

genases may be used. Many dehydrogenases are known (see for example Enzyme

Nomenclature 1992, Academic Press Inc.) and their properties can be found from the

10   literature or determined by simple spectrophotometric assays (see, e.g. Bergmeyer

[1974]). Besides naturally occuring enzymes with the desired pyridine nucleotide

specificities, the invention also includes the use of genetically engineered enzymes with

altered pyridine nucleotide specificities. As an example of cofactor specificity changes,

see e.g. Chen *et al.* (1994) and Chen *et al.* (1997).

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The catalytic activities responsible for reactions (1) and (2) may occur in the same

polymeric protein or even in a single polypeptide chain or be combined into such a

polymeric protein or single polypeptide chain, for example by genetic engineering. The

invention may also be realised by overexpressing a dehydrogenase that operates

20   effectively with both pyridine nucleotide systems. Dehydrogenases that accept both

pyridine nucleotides are known and include isozymes of glutamate dehydrogenase (EC

1.4.1.3), aldehyde dehydrogenase (EC 1.2.1.5) and alcohol dehydrogenase (EC 1.1.1.71).

Little is known about how their activities are regulated *in vivo* so that they do not

disturb the concentrations of pyridine nucleotides.

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In its second embodiment the invention provides a microorganism which is transformed

with at least one recombinant DNA molecule encoding or otherwise causing the

expression of at least one enzyme that catalyses at least one step of a cyclic series of

reactions in which NADP is reduced to NADPH and NADH is oxidised to NAD, or

30   *vice versa*. Reactions (3) to (5) show one such cycle:

(3) $NADP + SH_2 \rightleftharpoons S + NADPH$

(4) $S + X \rightleftharpoons Z + Y$

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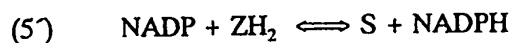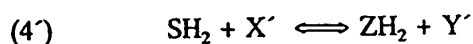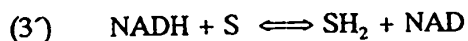$$(5) \qquad\qquad NADH + Z \iff SH_2 + NAD$$

In the direction written, reactions (3) to (5) convert NADP *plus* NADH to NADPH *plus* NAD, and in the opposite direction they carry out the opposite transformation. The

5   enzymes catalysing reactions (3) and (5) are again a pair of dehydrogenases with a common substrate (SH_2) but opposite coenzyme specificities, as in the first embodiment of the invention, but now the reaction products (S and Z) are different. For the purposes of this invention, reaction (4) can be a series of steps instead of a single step, provided only that S is converted into Z without a net change in NAD or NADP. Thus, this series

10   of reactions also tends to equilibrate the NAD/NADH and NADP/NADPH couples in the same way as reactions (1) and (2), except that this is now coupled to the transform-ation of X $\rightleftharpoons$ Y. Thus, at equilibrium it is not necessarily the case that the NAD/NADH and NADP/NADPH ratios will be nearly equal: instead they will also depend on the equilibrium between X and Y. An example of this embodiment of the

15   invention is provided by malic enzyme, pyruvate carboxylase and malate dehydrogenase, which catalyse the following reactions:

(6) *malic enzyme*:   malate + NADP $\rightleftharpoons$ pyruvate + CO_2 + NADPH

(7) *pyruvate carboxylase*:   pyruvate + CO_2 + ATP $\Rightarrow$ oxaloacetate + ADP + Pi

20 (8) *malate dehydrogenase*: oxaloacetate + NADH $\rightleftharpoons$ malate + NAD

In reactions (6) to (8), pyruvate + CO_2 correspond to S in reactions (3) to (5), oxaloacetate corresponds to Z and ATP and ADP + Pi, respectively, correspond to X and Y.

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In reactions (3) to (5) the reduced substrate, SH_2, is common to the two dehydrogenases. The invention can also be practised by transforming a microorganism with one or more enzymes to create a cyclic series of reactions (3´) to (5´) in which the oxidised form of the substrate is common to the two dehydrogenases:

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(3') $\quad$ NADH + S $\Longleftrightarrow$ SH$_2$ + NAD

(4') $\qquad$ SH$_2$ + X' $\Longleftrightarrow$ ZH$_2$ + Y'

(5') $\quad$ NADP + ZH$_2$ $\Longleftrightarrow$ S + NADPH

5　Once again, for the purposes of this invention the reaction (4') can be a series of steps instead of a single step, provided only that SH$_2$ is converted into ZH$_2$ without a net change in NAD or NADP.

Further, it is now an obvious extension of the invention to combine the cyclic schemes
10　(3) to (5) and (3') to (5') so that neither the oxidised nor the reduced forms of the substrates are common to the two dehydrogenases, but the oxidised forms of the substrates of the dehydrogenases can be interconverted by reactions with no net redox change and so can the reduced forms:

15　(3'') $\qquad\qquad\qquad\qquad$ NADP + SH$_2$ $\Longleftrightarrow$ S + NADPH

(4'') $\quad$ ZH$_2$ + Y' $\Longleftrightarrow$ X'+ SH$_2$ $\qquad\qquad$ S + X $\Longleftrightarrow$ Y + Z

(5'') $\qquad\qquad\qquad\qquad$ NADH + Z $\Longleftrightarrow$ ZH$_2$ + NAD
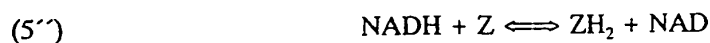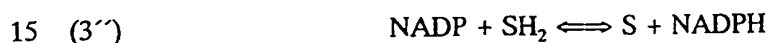
Thus, in its most general form the invention consists of transforming a micro–organism
20　with a gene causing expression of at least one enzyme catalysing one of the reactions (3'') to (5'') so that all these reactions can occur in the same cell compartment. When the reactions occur from left to right as written above, reducing equivalents are transferred from NADH through ZH$_2$ and SH$_2$ to NADP, reducing it to NADPH. When the reactions occur from right to left, NADH is formed at the expense of NADPH.
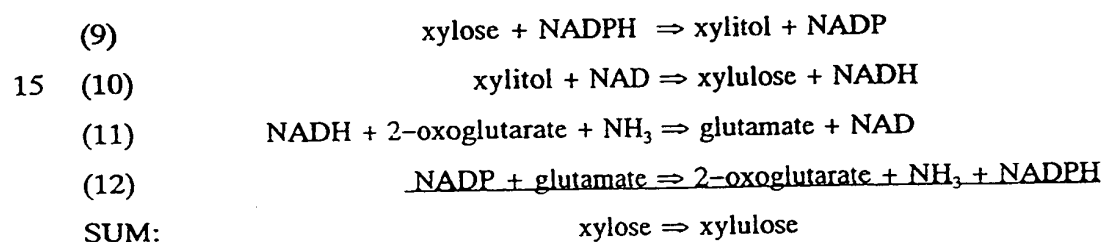
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Also in this embodiment of the invention, it is envisaged that a genetically engineered enzyme with changed coenzyme specificities can be used.

In the first specific aspect of the invention, the host microorganism carries an XR
30　enzyme which preferentially uses NADPH, and an XDH enzyme which preferentially uses NAD. The host microorganism can convert xylose into xylulose by these enzymes, but as described above, this process is inefficient and yields of ethanol on xylose are low or zero. An example of this aspect of the invention is provided in Example 8. An

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engineered strain of *S. cerevisiae* carrying genes for XR and XDH, and xylulokinase (XK), is transformed with a multicopy plasmid carrying the gene *GDH2* encoding the NAD–dependent glutamate dehydrogenase from *S. cerevisiae*, and a marker gene. Transformants are selected by means of the marker gene. The transformants ferment

5   xylose to ethanol more efficiently than the non–transformed host yeast, in particular with a higher yield of ethanol on xylose, or less CO_2 production or both. Depending on the chosen process conditions, the improved efficiency can also be realised in other ways, such as an increased volume productivity or enhanced specific rate. Enhanced specific rate is especially significant in processes utilizing immobilised microorganisms,

10   where there is an upper limit to the amount of biomass that can be held by the carrier material. The increased efficiency may be explained by the following sequence of reactions:

(9)   xylose + NADPH $\Rightarrow$ xylitol + NADP

15   (10)   xylitol + NAD $\Rightarrow$ xylulose + NADH

(11)   NADH + 2-oxoglutarate + NH_3 $\Rightarrow$ glutamate + NAD

(12)   NADP + glutamate $\Rightarrow$ 2-oxoglutarate + NH_3 + NADPH

SUM: xylose $\Rightarrow$ xylulose

20   Thus, the redox imbalance is avoided, and a smooth conversion of xylose to xylulose can take place. The flux through decarboxylation reactions, such as G6PDH and isocitrate dehydrogenase to regenerate NADPH is decreased, with decreased CO_2 production, and the fermentation occurs efficiently, and without aeration. Rather surprisingly, xylitol production was also increased by about 25 % in the strain

25   transformed according to the invention with GDH2 by comparison to the control strain. This increase in xylitol is discussed below.

In this example, the host microorganism had already been transformed with genes encoding XR, XDH and XK. It is not a requirement of the invention that the host organism is itself a transformant. It is remarkable that the invention causes a substantial

30   increase in ethanol yields with the host microorganism of Example 8, because the XR in this host is the enzyme from *P. stipitis*, which is able to work with NAD(H) although it has a preference for NADP(H) (Verduyn *et al.* [1985]). Thus, this aspect of the

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invention is realised even with an XR that can use NADH. A more substantial effect can occur when the host organism contains an XR with higher specificity for NADP(H), such as when the *S. cerevisiae* open reading frame XHR 104w encoding XR activity is highly expressed. Furthermore, it has been claimed that transformation of yeasts with an
5   gene encoding XK improves the efficiency with which they ferment xylose to ethanol (Ho and Tsao, WO 95/13362). It is notable that the present invention causes an improved fermentation of xylose to ethanol even when the host organism contains elevated levels of XK. However, the invention provides improved fermentation of xylose to ethanol also in host organisms that have not been transformed with a gene
10   encoding XK. It is now known that yeasts such as *S. cerevisiae* and *Schiz. pombe* contain homologous genes encoding XDH, XK and (*S. cerevisiae*) XR. The ability of a host yeast that does not contain any heterologous gene for XR, XDH or XK to ferment xylose to ethanol can also be usefully enhanced by transformation according to the present invention.
15

Various enzymes, including isomerases and epimerases, are known that interconvert the different pentose sugars and different pentose phosphates. It will be understood by a person skilled in the art that the present invention provides a general method to improve the efficiency of ethanol production, not only from xylose but also from other pentoses.
20

It will be understood by a person skilled in the art that similar beneficial effects can be obtained by using other pairs of dehydrogenases according to the first embodiment of the invention described above. For example, instead of transforming *S. cerevisiae* with *GDH2* so that both NAD- and NADP-linked glutamate dehydrogenases are adequately
25   expressed in the cytosol, the same effect can be achieved by transforming the yeast with one or both members of another pair of dehydrogenases that share the same substrates but use different pyridine nucleotides, provided that both of the enzymes are reversible, or at least that they catalyse the reactions in the directions shown in equations (11) and (12). For example, most NAD-linked isocitrate dehydrogenases are allosteric enzymes
30   that cannot catalyse the reductive carboxylation of 2-oxoglutarate (corresponding to reaction (11)), but only the oxidative decarboxylation of isocitrate, and would therefore be unsuitable for this aspect of the present invention. However, several other pairs of dehydrogenases can be used, including alcohol and aldehyde dehydrogenases. Appropri-

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ate information can be obtained from the literature or readily determined by testing with simple spectrophotometric assays. The activities of dehydrogenases can be easily measured in both directions, provided that they are reversible, by following the appearance or disappearance of NAD(P)H in the presence of the appropriate substrates
5   to determine whether candidate enzymes catalyse the reactions required to relieve the coenzyme imbalance.

According to the second embodiment of the present invention, similar beneficial effects can be obtained by transforming *S. cerevisiae* with a recombinant DNA molecule
10 encoding an NAD(P)-linked malic enzyme. *S. cerevisiae* already contains pyruvate carboxylase and malate dehydrogenase. The yeast can now catalyse the reactions (6) to (8) as shown above, resulting in the conversion of NADP *plus* NADH into NADPH *plus* NAD. Example 14 illustrates the beneficial effects of this transformation. Compared to the control strain, the strain overexpressing the gene for malic enzyme
15 exhibited a 55 % greater specific rate of xylose utilization and 20 % and 25 % greater specific rates of ethanol and xylitol formation.

The increased rates of xylitol formation observed in Examples 8 and 14 by strains constructed according to the first and second embodiments of the invention were
20 unexpected, because these particular strains contained also heterologous XDH and XK. XDH and XK are known to assist the conversion of xylose to ethanol, so these strains were a good test to show that the present invention can further improve xylose fermentation to ethanol under realistic conditions. XDH and XK are expected to facilitate the conversion of xylose-derived xylitol to xylulose-5-phosphate, so it is
25 significant that even in the presence of these two enzymes, transformation according to the present invention increased xylose uptake by more than it increased ethanol production, the difference appearing as an increase in xylitol production. Xylitol is an attractive product, either by itself or together with ethanol. Transformation according to the invention of strains that do not contain heterologous XDH and XK is expected to
30 provide at least as good improvements in xylitol production as those achieved in Examples 8 and 14.

Yeasts and other microorganisms produce other pentitols, in particular arabitol and

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ribitol, as well as xylitol. These can be produced from other natural pentoses (e.g. arabinose) that occur in raw materials, or by metabolic intercoversions of pentitols that often proceed through dehydrogenase reactions with differing coenzyme specifity (see, e.g., Chiang & Knight [1960]). In either case, similar redox balance problems occur as

5   those described above, and improved productivities and yields can be obtained by practise of the present invention.

It will be understood by a person familiar with the art that because it exploits fundamental biochemical principles the present invention has very broad application and

10   can be practised in other microorganisms as well as S. cerevisiae. Example 18 illustrates that transformation of Schiz. pombe with the gene encoding malic enzyme according to the invention improves the efficiency of the fermentation of xylose to ethanol. Volumetric and specific productivities were significantly enhanced also with this microorganism, and importantly it was able to maintain its biomass and metabolic

15   capacity under the process conditions, whereas the biomass of the control strain decreased relatively rapidly.

In another specific aspect of the invention, the host microorganism ferments hexose sugars to ethanol. Because the microorganism grows during the fermentation it produces

20   excesses of both NADH and NADP (Oura, [1972]). With the non–transformed microorganism, ethanol production is accompanied by glycerol production, which is required to reoxidise the excess NADH, and by the production of more than one mole of CO_2 per mole of ethanol, which is required to reduce the excess NADP. These reactions decrease the yield of ethanol on fermentable carbohydrate. With the transformed microorganism,

25   the yield of ethanol on fermentable carbohydrate is increased compared to that of the non–transformed microorganism. Illustrations of this aspect of the invention are provided in Examples 9 and 13.

In Example 9 the yeast S. cerevisiae is transformed with a multicopy plasmid

30   comprising the gene GDH2 encoding the NAD–dependent glutamate dehydrogenase from S. cerevisiae and a marker gene. The transformants are selected by means of the marker gene. The transformants ferment glucose to ethanol with improved efficiency, in particular an improved yield of ethanol on fermentable carbohydrates and with

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decreased production of some unwanted side products, including CO_2. Depending on the

chosen process conditions, the improved efficiency can also be realised in other ways,

such as an increased volume productivity or increased specific rate. This may be

explained by the following sequence of reactions that converts substantial parts of the

5   excess NADH and NADP into NAD and NADPH without unwanted consumption of

fermentable sugars:

NADH + 2-oxoglutarate + NH_3 ⇒ glutamate + NAD

NADP + glutamate ⇒ 2-oxoglutarate + NH_3 + NADPH
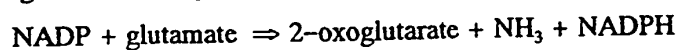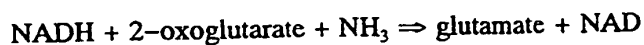
10

In Example 13, *S. cerevisiae* is transformed with a gene encoding malic enzyme

according to the second embodiment of the invention. Significant advantages are

achieved, including again decreased production of (undesired) biomass and increased

specific rate of ethanol production.

15 ·

It will be understood by a person skilled in the art that similar beneficial effects on

hexose fermentation to ethanol can be obtained by using other pairs of dehydrogenases

according to the first embodiment of the invention disclosed above or by using other

appropriate enzymes according to the second embodiment of the invention and that the

20   invention can be practised with other microorganisms as well as *S. cerevisiae*.

It is a significant part of the invention that the same transformation that increases the

efficiency of xylose fermentation to ethanol (Examples 8, 14 and 18) also increases the

efficiency of hexose fermentation to ethanol (Examples 9 and 13). Thus, the invention

25   simultaneously provides improved utilization of both glucose and xylose, which are

major sugars derived from many renewable biomasses, such as agricultural and forest

materials and urban waste.

For industrial production of ethanol, the present invention is preferably practised using

30   an industrial strain, e.g. a distiller's or brewer's yeast, or a wine yeast, or a strain of

Schiz. pombe used for rum production. Methods to transform industrial yeasts, which are

often polyploid and lack auxotrophic markers are well known. An early review of such

methods was made by Knowles and Tubb (1987). The industrial strain transformed

according to the invention is then used for example in the same industrial fermentation
process as the non-transformed strain and provides the advantages disclosed above,
such as increased yield, increased productivity and decreased undesirable side-products.
Compared to the non-transformed strain, the transformed strain can also be used
5   advantageously for the economic fermentation of a wider range of raw materials, such
as raw materials with high levels of pentoses, pentose polymers or both.

In a third specific aspect of the invention a microorganism that overproduces an amino
acid such as alanine, valine, leucine, isoleucine, serine, threonine, lysine, arginine,
10 tryptophan, cysteine, methionine or proline is transformed with at least one recombinant
DNA molecule encoding at least one of a pair of dehydrogenases with opposite pyridine
nucleotide specificities (i.e., according to the first embodiment of the invention), or with
at least one recombinant DNA molecule encoding at least one enzyme that catalyses at
least one step of a cyclic series of reactions in which NADP is reduced to NADPH and
15 NADH is oxidised to NAD according to reactions (3) to (5), (3´) to (5´) or (3´´) to (5´´)
(i.e. according to the second embodiment of the invention). The transformed
microorganism can produce the desired amino acid with improved efficiency, in
particular with increased yield on carbon source, with increased productivity, with a
decreased requirement for aeration, with decreased production of carbon dioxide or with
20 several of these benefits.

For example, lysine is presently produced by microbial fermentation processes that are
mainly based on various Corynebacteria, such as *Corynebacterium glutamicum* and
Brevibacterium flavum. Genetic techniques for these bacteria are well developed (see,
25 e.g., Follettie *et al.* [1991]; Follettie & Sinskey [1986]; Jetten *et al.* [1994]; Jetten &
Sinskey [1995]) and DNA vectors are available for the transformation of these produc-
tion organisms efficiently by either multicopy plasmids or chromosomal integration. For
example, available vectors include pAJ655, pAJ1844 and pCG11 for use with *C.
glutamicum*, *Brevibacterium* spp., and *Escherichia coli* or the pAJ440 plasmid vector for
30 use in *Bacillus subtilis*, *Brevibacterium* spp., and *C. glutamicum*, or the pMS2 plasmid
vector for use in *Rhodococcus* spp., *Corynebacterium* spp., and *E. coli*. The effects of
transformation of lysine-producing strains according to the present invention can be
realised in strains such as ATCC 31269, ATCC 21253, ATCC 21800, ATCC 21801 or

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ATCC 21086 or in other strains that overproduce lysine or other amino acids and are used industrially.

This aspect of the invention is illustrated in Examples 19 to 23. *Corynebacterium*
5   *glutamicum* was transformed with a gene from *Peptostreptococcus asaccharolyticus*
encoding an NAD–dependent glutamate dehydrogenase. The transformed
Corynebacterium glutamicum shows NAD–linked glutamate dehydrogenase activity, and
this organism naturally possesses NADP–linked glutamate dehydrogenase activity. The
transformed organism therefore possesses a dehydrogenase pair according to the first
10 embodiment of the invention that can convert NADP plus NADH into NAD plus
NADPH. Unlike some bacteria, *Corynebacterium glutamicum* does not contain
NADP/NADH transhydrogenase, so the sequential operation of the two glutamate
dehydrogenases provides the bacterium with the novel means to equilibrate the
NAD/NADH and NADP/NADPH coenzyme couples. It is well known that the synthesis
15 of lysine (and most other amino acids) produces NADPH, and that when lysine is
overproduced in large amounts the requirement for reoxidation of NADPH to NADP
can limit the amino acid production. It is also known that under the cultivation
conditions used in Example 23 production of lysine does not begin while threonine is
still present in the medium and that yields are relatively low until the bacteria stops
20 growing (Vallino, J.J. [1991]; see expecially pages 207 to 213). Surprisingly,
Corynebacterium glutamicum transformed according to the invention already produced
large amounts of lysine while threonine was still present and before the bacterium had
reached even 25 % of the expected biomass yield. These examples disclose that the
present invention can be practised with advantage also in bacteria as well as fungi and
25 for improving the production of amino acids as well as non–nitrogenous compounds
such as ethanol and xylitol.

For the industrial production of amino acids, a bacterial strain that overproduces one or
more amino acids is transformed according to the invention. The transformed strain can
30 be used in the same industrial processes as the parent non–transformed strain. For
example, raw materials can be used that comprise any of a variety of sugars or organic
acids such as citric, succinic or fumaric acid as carbon source and ammonia, ammonium
salts or inexpensive protein hydrolysates as nitrogen source. Trace organic (e.g.

thiamine) and inorganic (e.g. iron and manganese salts) materials can be added in the same way as for the original process with the parent non-transformed strain. Compared to the non-transformed strain, the strain transformed according to the present invention provides the advantages disclosed above, such as increased yield, increased productivity,

5 decreased oxygen requirement.

An NAD-linked glutamate dehydrogenase can also be introduced to *Corynebacterium glutamicum* (and other Corynebacteria spp.) by any method well known in the art. For example, the gene from *Peptostreptococcus asaccharolyticus* has been transferred to *C.*

10 *glutamicum* under a Tac promoter by Marx *et al.* (1999). Significantly, these workers used a strain of *Corynebacterium glutamicum* from which the gene coding for NADP-linked glutamate dehydrogenase was first deleted, whereas the present invention teaches the simultaneous presence of NADP-linked and NAD-linked glutamate dehydrogenases. The invention can also be practised by transforming bacterial strains that overproduce

15 other amino acids than lysine with this or another gene encoding NAD-linked glutamate dehydrogenase or with the gene for some other enzyme that causes the functional coupling of two dehydrogenases with different specificities for the pyridine nucleotide couples.

20 Polyhydroxyalkanoates (PHAs) are commercially produced to make biodegradable plastics, but prices are too high for widespread use except where this is enforced by legislation (e.g. in Germany). It is therefore desirable to improve the efficiency of the microbial processes producing PHAs. In the biosynthesis of PHAs, glucose is metabolised to acetylCoA, producing 2 NADH molecules/acetylCoA molecule, and the

25 acetylCoA is then condensed to acetoacetylCoA which is reduced by NADPH to 3-hydroxybutyrylCoA. Synthesis of each molecule of 3-hydroxybutyrylCoA therefore produces 4 molecules of NADH and requires 1 molecule of NADPH. The 3-hydroxybutyrylCoA is then polymerised to polyhydroxybutyrate (PHB) or copolymerised with other acylCoAs such as propionylCoA to form mixed PHAs. The

30 requirement for one NADPH molecule and production of 4 NADH molecules per monomer unit means that microorganisms synthesising PHAs need to divert part of their carbon flux through reactions such as glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase in order to generate NADPH, with consequent excess production of CO_2

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and waste of carbon source, as explained above. At the same time, NADH must be reoxidised, causing either further carbon losses or increased oxygen demand or both. This waste can be decreased by transforming the production microorganism according to the present invention, so providing it with a novel mechanism that converts part of the

5   excess NADH produced into the NADPH required (for reviews, see e.g. Anderson and Dawes [1990]; Poirier *et al.* [1995]).

In this aspect of the invention, a microorganism that produces one or more PHAs is transformed according to the first or second embodiments, and the transformed

10   microorganism is used to ferment glucose in the presence or absence of organic acids such as propionic or valeric acids. Compared to the non-transformed parent organism, the organism transformed according to the invention provides the advantages disclosed above, such as producing increased yields of PHA on glucose, increased productivities and decreased formation of unwanted sideproducts, such as CO_2. Example 24 illustrates

15   how this can be done. In this example the production microorganism is a recombinant strain *Saccharomyces cerevisiae* transformed with the PHB synthase and reductase genes from *Alcaligenes eutrophus*. However, it is not necessary that a recombinant yeast is used. Any microorganism producing PHAs can be used, e.g. *Alcaligenes eutrophus* or *Pseudomonas oleovarans*, or engineered bacterial strains that over-produce PHAs. The

20   microorganism transformed according to the present invention is then used to ferment glucose with or without organic acids under essentially the same process conditions as used for the non-transformed parent organism.

In another aspect of the invention a production microorganism is transformed according

25   to the invention and under the conditions of a biotechnological process, the transformed microorganism maintains a higher level of the metabolic capacity required for the said process than does the corresponding non-transformed microorganism. Example 14 illustrates this aspect. *S. cerevisiae* transformed according to the invention with a gene expressing malic enzyme is used to ferment xylose to ethanol under anaerobic

30   conditions, which are advantageous because they prevent a decrease caused by oxidative processes in the yield of ethanol. Under these process conditions the biomass of the control strain decreases with time during operation of the process with a corresponding loss of its metabolic capacity to convert substrate (xylose) into product (ethanol). In

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practice this would lead to inconvenient and expensive process requirements such as repeated addition of more production organism. However, the organism transformed according to the invention is able to maintain its biomass and the metabolic capacity required by the process (Fig. 8). Presumably this is because the transformed organism

5   is able to obtain sufficient energy from the biotransformation of the process (in the case of Example 14, the conversion of xylose to ethanol) to maintain its own integrity and metabolic capacity, whereas the non-transformed organism cannot. Under the conditions of Example 14, with a unicellular production microorganism and a homogenous liquid medium, it was easy to demonstrate the decrease in biomass of the control micro-

10   organism and the maintenance of the biomass of the organism transformed according to the invention.

However, it is clear to a person familiar with the art first that in many processes, e.g., those employing inhomogenous media or filamentous fungi or both, it can be very

15   difficult to demonstrate the maintenance of biomass, and second that the significant parameter is not the biomass itself but its metabolic capacity to convert the substrate of the process into the product. In this connection metabolic capacity means the total ability of the production organism present in a process (e.g. per unit volume of a fermentor) to perform a particular set of biotransformations, namely those required by

20   the particular biotechnological process, and it can be measured by measuring the rate of conversion of process substrate into process product under standard conditions.

Other aspects of the invention include the transformation of production microorganisms that have been developed to overproduce nucleotides, lipids or secondary metabolites of

25   various types on an industrial scale. After transformation according to the above described embodiments of this invention, these microorganisms will provide increased yields of the desired commercial products, or increased specific production rates, decreased formation of undesirable side products (such as CO_2 or excess biomass) or several of these advantages simultaneously.

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These examples disclose how the efficiency of biotechnological processes can be improved by transforming production microorganisms with at least one recombinant DNA molecule encoding enzymes that facilitate oxidation-reduction reactions between

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the NAD(H) and NADP(H) coenzyme systems. The invention can be realised by trans-
forming the host with a single gene (e.g. *GDH2* in Example 3; *MAE1* in Example 12)
and using enzymes that are naturally expressed in the host under the specific production
conditions to complete the reaction schemes (i.e. reactions (1)+(2) or (3)+(4)+(5)

5   above). However, the invention can also be realised by transforming the host with more
than one DNA-molecule so that both reactions (1) and (2) or two or more of the
reactions (3), (4) and (5) (or (3'), (4') and (5'), or (3''), (4'') and (5'')) are performed
by enzymes expressed from transformed genes.

10   Besides *GDH2* and a gene encoding malic enzyme, genes encoding other enzymes can
be advantageous. Suitable enzymes (examples are given above) are known and their
genes have been cloned and can be found in data banks and obtained by PCR methods
well known in the art (Examples 6 and 7 and 10 confirm how readily this can be done
for genes from both yeast and filamentous fungi). A person skilled in the arts of

15   microbial physiology and metabolism can plan a metabolic scheme corresponding to
reactions (1) and (2), or (3) to (5), (3') to (5') or (3'') to (5''). Many such schemes
require well known enzymes. Data banks (e.g. Swissprot) can also be searched to find
the requisite enzyme or enzymes. Thus, for example, the following accession numbers
provide amino acid or nucleotide sequences corresponding to NADP-linked

20   dehydrogenases: P00369, P31026, P00370, P50216, P08200, P14941, P75214, P27800,
Q58820, P15719, P46919, P28861, L05667, U26463, YPL061w, P18819, M88600,
X75327 and P55804. These sequences can be used to clone the corresponding genes,
e.g., by PCR with sequence specific primers.

25   Other suitable enzyme activites can be found by carrying out appropriate enzyme assays
(see, e.g., Bergmeyer [1974], but other suitable assay systems can be readily designed
by a person skilled in the art) on extracts prepared from suitable organisms, including
bacteria, fungi and higher plants and animals. The responsible protein can then be
purified by standard methods, and antibodies prepared against it or amino acid sequence

30   data obtained from it. The gene encoding the protein can then be cloned by standard
methods such as using antibodies to screen expression libraries or oligonucleotides
designed from the amino acid sequences to act as primers in PCR cloning or hybridiza-
tion probes to screen gene banks.

25

Suitable new enzyme activities and their genes can also be found by exploiting data

bank information in other ways that are familiar and routine to people skilled in the art.

For example, alignment of several sequences encoding malic enzymes reveals a so

called "malic enzyme signature", which allows the preparation of oligonucleotide

5   mixtures that can be used, for instance, in PCR cloning of genes encoding malic

enzymes in other organisms, as is described in Example 7 for the malic enzyme of

Aspergillus nidulans.

It is well known to a man skilled in the art that during the cloning of genes by PCR,

10   point mutations can occur and be perpetuated by the amplification technique. Small

sequence differences between the same genes in different strains of the same organism

can also occur naturally. Many of these changes have no significant effect on the

function of the encoded protein and are therefore called neutral mutations. For example,

two point mutations in the gene encoding malic enzyme were observed in Example 10,

15   but did not significantly affect the activity of the encoded malic enzyme. Such neutral

mutations can also be introduced deliberately. Our invention encompasses the use of

such neutral variants of genes as well as the natural genes.

According to the present invention, the host organism is transformed in such a way that

20   the reactions (1) and (2) in the first embodiment of the invention or the reactions (3) to

(5) (or (3') to (5') or (3'') to (5'')) in the second embodiment occur simultaneously

preferably in the same subcellular compartment, preferably the cytosol. The restriction

that the reactions occur in the same compartment is not absolute because reactions (4'')

can include transport or shuttle reactions moving metabolic intermediates between

25   subcellular compartments. The invention teaches that the transforming gene can be

modified if necessary to cause expression in the appropriate compartment and under the

physiological conditions prevailing during the desired production process. So called

"signal" or "targeting" sequences are known that usually encode relatively short N–

terminal or C–terminal amino acid sequences that direct proteins to specific com–

30   partments such as mitochondria, peroxisomes or periplasmic space (McAlister–Henn *et*

al. [1995]). These sequences can be readily removed or added to genes by standard

techniques of genetic engineering to cause the desired enzymes to be expressed in the

desired compartment. Malic enzyme expressed from the complete *MAE1* gene (as in

Examples 13, 14 and 18) is an example of an enzyme with a mitochondrial targeting sequence (Boles *et al.* [1998]), so that at least some of its activity is expected to be located inside mitochondria. When the gene is strongly expressed, as in Examples 13 and 14 it is likely that a part of the enzyme remains in the cytosol, where also the yeast

5   malate dehydrogenase and pyruvate carboxylase are found. If it is desired to express the malic enzyme (or some other enzyme with a mitochondrial targeting sequence) only in the cytosol, then the targeting sequence can be removed by truncating the gene appropriately. In addition, enzymes subject to catabolite inactivation can be modified to slow or prevent this regulatory circuit (Minard and McAlister-Henn [1992]).

10

The present invention can also be practised by transforming a microorganism with a recombinant DNA molecule so that the natural promoter of a host gene encoding a suitable enzyme that catalyses one of the reactions (3´´) to (4´´) is replaced by another promoter that can cause stronger expression or expression under different physiological

15   conditions than the said natural promoter. It is not necessary that the transforming DNA molecule contains a nucleotide sequence encoding a complete functional enzyme. For example, the beneficial effect can be obtained by transforming the host *S. cerevisiae* with a DNA molecule that only replaces through recombination *in vivo* the natural promoter of the host's *GDH2* with a promoter such as *PGK* or *ADH*, so that the host's

20   NAD-dependent glutamate dehydrogenase is constitutively expressed. When the host is transformed with a gene from another organism, it is desirable to use a promoter derived from the host.

For many production fungi and bacteria suitable promoters are known. Examples

25   include the promoter of the *S. cerevisiae PGK, ACT, ENO1, GAPDH, MET* genes, such as *MET25* and *ADH* promoters, and modified versions thereof (e.g. Ruohonen *et al.* [1995]; Beier and Young [1982]). It is envisaged in the invention that, in the case of *S. cerevisiae* for example, use of these promoters can be advantageous even when the transformed gene or genes are obtained from yeast. They can be advantageous

30   especially when the genes are to be integrated into the host's genome, because these promoters are known to cause adequate expression over a range of physiological conditions. For example, the so called middle length *ADH1* promoter causes efficient expression in *S. cerevisiae* under both fermentative and gluconeogenic growth condi-

tions. However, adequate expression of the transformed gene or genes can also be obtained with the genes' natural promoters, for example, by transformation with a multicopy plasmid, as disclosed in Examples 3 to 5. Efficient expression under desired physiologoical conditions can also be obtained by modifications of the promoter in question or by modifications of the transacting regulatory mechanisms (negative or positive) involved in the expression of the particular gene.

When foreign genes are transformed into an organism, it is desirable to transform with a DNA sequence without introns, obtained for example from cDNA or by artificial synthesis.

Any method known in the art for introducing or transforming genes into the host is suitable for this invention and various types of vectors can be used, including autonomously replicating plasmid vectors or artificial chromosomes. Methods described in the art to integrate single or multiple copies of transforming genes into chromosomes in functional, expressible forms are also suitable for this invention. Examples of such methods for yeast, filamentous fungi and Corynebacteria, and other microorganisms have been described. An appropriate marker gene can be included in the transforming vector so that transformants can be easily selected. Co-transformation with a second vector containing a selectable marker gene can also be used. A wide range of marker genes is known. Transformants can also be selected by expression of a desired pheno-type, such as enhanced ability to grow on xylose under anaerobic conditions (see Example 8).

It is envisaged in the invention that it can be advantageous in some cases to cause expression of the transformed genes only under specific culture conditions. For example, it can be useful first to grow the organism to a certain cell density, and then cause expression of the transforming gene. Promoters are known that can be induced by changes in temperature or pH, by particular carbon or nitrogen sources or by the presence or absence in the medium of certain organic or inorganic substances, such as phosphate or copper. Examples of yeast promoters that have been used for such inducible expression include *GAL1*, *GAL10*, *CUP1* and *PHO5*.

28

The present invention is further illustrated by the following Examples which describe construction of the production strains of the invention, as well as their use in the above indicated specific aspects of the invention. If not otherwise indicated, all biotechnological procedures are carried out using methods conventional in the art.

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Example 1. Construction of the integrant strain with the *XYL1* and *XYL2* genes of *Pichia stipitis* encoding xylose reductase and xylitol dehydrogenase

The pMA91 (Mellor *et al.* [1983]) based yeast expression vector pAOS66 (Figure 1)
10 containing the *XYL1* under the *PGK1* promoter and the *XYL2* under the modified *ADH1* promoter (Ruohonen *et al.* [1995]) was digested with *Hind*III to isolate the 2.8 kb expression cassette carrying the *XYL1* gene between the promoter and terminator of *PGK1* and with *Bam*HI to isolate the 2.2 kb expression cassette carrying the *XYL2* gene between the modified *ADH1* promoter and *ADH1* terminator. Plasmid B955 (Toikkanen
15 *et al.* [1998]) was used to construct the integration cassette. B955 is the Bluescript SK bacterial cloning vector (Stratagene) carrying two fragments of the *URA3* gene (encoding orotidine–5'–P decarboxylase, Rose *et al.* [1984]); base pairs 71–450 and 781–1135 from the encoding region of the gene at *SacI–XbaI* sites and *XhoI–Asp718* sites, respectively, of the polylinker region. The remaining polylinker sites *Hind*III and
20 *Bam*HI in the cloning vector were used for introducing the *XYL1* and *XYL2* expression cassettes between the two *URA3* fragments by sticky–end ligations. The resulting construction (5' *URA3* 71–450 bp – *XYL2* expression cassette 3'–5' – *XYL1* expression cassette 5'–3' – *URA3* 781–1135 3') was released from Bluescript SK by *SacI–NsiI* digestion and isolated from an agarose gel. One μg of the fragment was used to
25 transform the yeast strain CEN.PK2 (VW–1B) (*MATα leu2–3,112 ura3–52 trp1–289 his3–Δ1 MAL2–8^c SUC2*) (Boles *et al.* [1996]) by the LiAc transformation procedure (Hill *et al.* [1991], Gietz *et al.* [1992]). The strain CEN.PK2 (VW–1B) is called "strain H1346" by us, and it has a VTT strain collection number VTT C–98304.

30 The integration strategy is based on the toxicity of 5–FOA (5–fluoro–orotic acid) to the yeast cells (Boeke *et al.* [1984]). Wild type cells convert 5–FOA to 5–FUMP (5–fluoro–uridine monophosphate), a potent inhibitor of thymidylate synthetase. Thus only *ura3* (and *ura5*) mutants can grow in the presence of 5–FOA, as long as uracil is

29

provided for the mutant strain. Integration of the above described fragment into the
URA3 locus disrupts the wild type gene and the strain becomes uracil auxotrophic,
allowing it to grow on 5-FOA plates.

5   The correct, functional integration was verified by Southern blotting, by measuring the
XR and XDH activities in cell extracts and by showing that the integrant strain only
grew on xylose in shake flask cultivations, in contrast to the non-transformed CEN.PK2
(VW-1B) strain. The integrant strain was named as H1469.

10   **Example 2. Cloning of Saccharomyces cerevisiae xylulokinase gene (SGD no. YGR
     194C)**

The xylulokinase gene (XK) was amplified from total DNA of wild type yeast strain by
(SEQ ID NO:6)
PCR, using forward primer 5' CCA GTG ATA TCG AGG ATG AGA TTA GTA C 3'
(SEQ ID N:7)
15   and reverse primer 5' CCA GTG ATA TCT GTA CTT GTC AGG GCA T 3'. Both
primers contain an EcoRV restriction site at the 5' end. PCR reaction conditions were:
94°C 3' hot start; 94°C 1', 55°C 1', 72°C 2', 30 cycles, 72°C 10' final extension. The
PCR product was digested with EcoRV and purified from an agarose gel. The XK
fragment was ligated into the vector B609 (Bluescribe M13; Stratagene, with the
20   modified ADH1 promoter and ADH1 terminator) which had been treated with Klenow
enzyme to make blunt ends. Orientation of the fragment was checked with BglII and
EcoRI enzymes. A clone with the right orientation was digested with BamHI and the
fragment was purified from an agarose gel. The BamHI fragment was cloned to BamHI
site of YEplac195 yeast expression vector (Gietz and Sugino [1988]).

25

**Example 3. Cotransformation of the integrant strain H1469 with the genes
encoding xylulokinase (XK) and NAD-dependent glutamate dehydrogenase (NAD-
GDH) on two separate multicopy expression vectors**

30   Two yeast expression vectors, the above described YEplac195 carrying the gene
encoding the xylulokinase and YEplac181 carrying the gene GDH2 encoding the NAD-
dependent glutamate dehydrogenase (Boles et al. [1993]) were cotransformed into
H1469 integrant strain. YEplac195 vector was selected for by omitting uracil and

30

YEplac181 by omitting leucine from the growth medium. Plasmid rescue from the yeast transformants verified the integrity of the two expression plasmids. The strain carrying both XK and NAD–GDH encoding genes was named as H1803 (VTT C–98302). A control strain carrying the gene encoding XK and YEplac181 control plasmid without

5   *GDH2* was named as H1805 (VTT C–98303).

Example 4. Transformation of the integrant strain H1469 with the gene encoding NAD–dependent glutamate dehydrogenase on a multicopy expression vector

10   The above described plasmid YEplac181 carrying the gene *GDH2* encoding the NAD–dependent glutamate dehydrogenase was transformed into the integrant strain H1469. Selection of the transformants was as mentioned above. Plasmid rescue from the yeast transformants verified the integrity of the expression plasmid. The strain carrying the gene encoding NAD–GDH was named as H1795 (VTT C–98300). A control strain

15   carrying the YEplac181 control plasmid was named as H1797 (VTT C–98301).

Example 5. Transformation of the yeast strain H1346 (CEN.PK2 (VW–1B)) with the gene encoding NAD–dependent glutamate dehydrogenase on a multicopy expression vector

20

The above described plasmid YEplac181 carrying the gene *GDH2* encoding the NAD–dependent glutamate dehydrogenase was transformed into the CEN.PK2 (VW–1B) (=H1346) strain. Selection of the transformants was as mentioned above. Plasmid rescue from the yeast transformants verified the integrity of the expression plasmid. The strain

25   carrying the gene encoding NAD–GDH was named as H1791 (VTT C–98298). A control strain carrying the YEplac181 control plasmid was named as H1793 (VTT C–98299).

Example 6. Cloning of the open reading frame YKL029C encoding the malic
30   **enzyme homologue from *Saccharomyces cerevisiae***

The malic enzyme has been characterized from *S. cerevisiae* (Fuck *et al.* [1973]). Analysis of the yeast genome revealed one open reading frame (ORF YKL029C) with

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homology to the gene encoding malic enzyme from *Schizosaccharomyces pombe*

(Viljoen *et al.* [1994]). The *S. cerevisiae* ORF YKL029C was amplified from the yeast

chromosomal DNA by PCR using forward primer 5' CAT GCT AAG CTT CTA GAA
(SEQ ID NO:8)

TGC TTA GAA CCA GAC TA 3' and reverse primer 5' GAT GCT AAG CTT CTA
A (SEQ ID NO:9)

GAT GGT TAT GCT TCG TCT AC 3'. Both primers contain *Hind*III and *Bgl*II
A

restriction sites at the 5' end. PCR reaction conditions were: 94°C 3' hot start; 94°C

1', 40°C 1', 72°C 2', 30 cycles, 72°C 10' final extension. The DNA fragment obtained

was of expected size. The PCR fragment was digested with the appropriate restriction

enzyme (*Bgl*II) to allow its cloning between the promoter and the terminator of *PGK1*

in the yeast expression vector pMA91.

Example 7. Cloning of the gene encoding the malic enzyme from the filamentous

fungus *Aspergillus nidulans*

So far all the genes encoding the malic enzymes cloned from different organisms

contain a DNA sequence coding for the "malic enzyme signature". It is a highly
(SEQ ID NO:10)

conserved, unique amino acid sequence (FNDDIQGTGAVVMASLI) of this particular
A

protein (Prosite: PDOC00294). The signature allows specific degenerated primers to be

planned for cloning of any particular gene encoding a malic enzyme.

Degenerate primers were designed using the malic enzyme signature (region D) for the

3' end primer and a second homologous region of the protein, the region C for the 5'

end primer (Viljoen *et al.* [1994]). The forward primer was 5' GA(T/C) GTI GGI ACI
(SEQ ID NO:11)

AA(T/C) AA 3' and the reverse primer was 5' GTI CC(T/C) TG(A/G/T) AT(A/G)
A (SEQ ID NO:12)

TC(A/G) TC(A/G) TT(A/G) AA 3'. PCR reaction conditions were 94°C 3' hot start;
A

94°C 1', 37°C 1', 72°C 2', 7 cycles, 94°C 1', 40°C 1', 72°C 2', 25 cycles, 72°C 10'

final extension Chromosomal DNA of *Aspergillus nidulans* was used as the template in

the PCR reaction. A fragment of expected size was obtained (0.24 kb) and Figure 2

shows the alignment of the PCR product with some known malic enzymes. A partial
SEQ ID NO: 1

amino acid sequence of *A. nidulans* malic enzyme is given in Sequence No 1. The

partial sequence obtained allows the design of further primers, and can itself be used for

probing by Southern hybridisation to clone the whole malic enzyme from any DNA

bank (e.g. cDNA, chromosomal, lambda). The gene encoding the malic enzyme from

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Trichoderma reesei, another filamentous fungus can also be cloned. A partial amino acid sequence thereof is included in Figure 2, and given also in Sequence No. 2). *SEQ ID NO: 2*

Example 8.   Ethanol Production from Xylose

Part 1.   Shake flask cultivations

The strains H1803 and H1805 (see Example 3) were cultivated in the growth medium which was modified SC-URA-LEU (synthetic complete media, uracil and leucine omitted, Sherman *et al.* [1983]) and Yeast Nitrogen Base without Amino Acids (Difco) and the carbon sources D-glucose (20 g/l) or D-xylose (20 g/l). Cells were pre-grown in shake flasks in a medium containing glucose as a carbon source. Cells were collected by centrifugation and resuspended in a volume of 100 ml media containing xylose as a carbon source. Cells were kept at 30 °C in 100 ml Erlenmeyer flasks gently stirred with a magnetic rod. Anaerobiosis was achieved by using an airlock.

After two days' incubation with xylose the strain with elevated levels of NAD-GDH had produced 2.35 g ethanol / g dry weight, and the control strain produced 1.47 g ethanol / g dry weight. The amount of ethanol was measured enzymatically with the aid of an automated analyser (Cobas - Mira).

Glutamate dehydrogenase activity was measured in a crude yeast cell extract, which was obtained by vortexing 500 mg of fresh cells in 500 μl of 100mM Na-phosphate, pH 7.0 and 1 g glass beads (diameter 0.4 mm) for 15 minutes. The mixture was then centrifuged in a table top centrifuge and the supernatant assayed. 200 μl of a buffer containing 200 μM NAD(P)H and 100 mM Na-phosphate, pH 7.4 were mixed with 10 μl of the 20 fold diluted crude yeast extract. To start the reaction α-ketoglutarate (final concentration 10 mM) and ammonium chloride (final concentration 20 mM) were added. The rate of decreasing absorbance at 340 nm was measured and the activity was calculated from this. It is related to the protein content of the yeast extract as measured by a BIORAD protein assay. The NADP-GDH activity was measured using NADPH as a substrate and the NAD-GDH activity was measured with NADH as a substrate. The NAD-GDH activity was estimated 4 – 5 nkat/mg in the overexpression strain, and 0.04

33

nkat/mg in the control without *GDH2* overexpressed. The NADP–GDH activity was about 2 nkat/mg. All assays were performed in a Cobas – Mira automated analyser.

Part 2. Fermentor cultivations

5   Anaerobic xylose fermentation to ethanol was conducted in 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of *Saccharomyces cerevisiae*, designated as **H1803** and **H1805**. Both strains are derived from *S. cerevisiae* CEN.PK2 (VW–1B) (Boles *et al.* [1996]), and express xylose reductase (XR) and xylitol dehydrogenase (XDH) by chromosomal integration of the corresponding genes from

10   *Pichia stipitis*. In addition, both strains overexpress the native xylulokinase (XK) from a multicopy plasmid (YEplac195+*XK*). Strain H1803 contains an additional plasmid that expresses the NAD–glutamate dehydrogenase (NAD–GDH) from *S. cerevisiae* (YEplac181+*GDH2*), whereas strain H1805 contains only the cloning vector (YEplac181) and serves as a control strain. Omitting uracil (URA) from the growth

15   media can minimize plasmid segregation for the YEplac195 vector, and leucine (LEU) for YEplac181.

The seed cultures of strains H1803 and H1805 were routinely maintained on plates that were renewed every 2–3 weeks. The pre-inoculum was prepared by transferring a

20   single colony into a 250 ml Erlenmeyer flask that contained 50 ml of modified synthetic complete medium without uracil and leucine (SC–URA–LEU) + 20 g/l of glucose (Sherman *et al.* [1983]). For each strain three identical flasks were prepared. The cells were grown overnight on a rotary shaker at 150 rpm and 30 °C, and the content of each flask was then transferred completely into a 3 l baffled flask that contained 500 ml of

25   SC–URA–LEU plus 50 g/l of glucose and grown aerobically at 150 rpm and 30 °C until glucose was exhausted (OD_{600}: 20–25).

Cells from the above cultivation (six flasks) were harvested by a 10 minute centrifugation at 4,500 rpm and 4°C, washed by a 0.1 M PO_4^{2-} buffer (pH=5.5) and

30   resuspended in the same buffer each to a final volume of 30 ml and subsequently transferred to the fermentor. The fermentation medium contained SC–URA–LEU + 10% xylose. The fermentor temperature was maintained at 30 °C, the pH was con-trolled at 5.5 by addition of 2 M NaOH, and the agitation was constant at 300 rpm.

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The cultivation was carried out under anaerobic conditions by flashing the headspace of the fermentor with nitrogen at a constant flow rate of 0.2 vvm. The offgas was connected via a multi-port valve to a Balzers quadrupole mass spectrometer (Sweden) for online analysis. Liquid samples were withdrawn from the fermentor at time intervals

5   to measure growth, substrate consumption, and the formation of extracellular products. For selected samples the activities of the NADP- and NAD-glutamate dehydrogenases were also measured by standard enzymatic techniques. Growth was monitored by measuring both the absorbance at 600 nm, as well as the dry cell weight (DCW) by filtration and subsequent drying to constant weight. Xylose, ethanol, xylitol, glycerol

10 and acetate present in the fermentor broth were separated on an HPX-87H column (55 °C), with 5 mM H_2SO_4 as eluent (0.6 ml/min), and quantified by refractive index (RI) detection. The amounts of ethanol, glycerol, xylitol and acetate were independently verified by appropriate enzymatic assays with the aid of an automated analyser (Cobas – Mira).

15

The growth and xylose consumption of strains H1803 and H1805 for the first 30 hours of cultivation are summarized in Figure 3. Both strains can consume xylose effectively at comparable rates, however, the NAD-GDH overexpressing strain (H1803) accumu- lates about 6% less biomass (7.13 vs. 6.72 g/l). The most remarkable difference

20 between the two strains is ethanol production. As illustrated in Figure 4, by the end of the 30-hour time period the *GDH2* strain accumulates about 1.02g ethanol per g DCW compared with 0.73 for the control strain. This represents an enhancement of specific ethanol production of approximately 40% for the *GDH2* strain (0.58 vs. 0.83 mmol/g- cell h). The volumetric productivity is also higher for the *GDH2* strain by about 30%

25 (3.94 vs. 5.20 mmol/l h). The corresponding yields of ethanol on xylose are 0.21 and 0.29 (mol/mol) for the control and *GDH2* strains, respectively. Unexpectedly, xylitol production was also elevated for the *GDH2* strain by about 25% as shown again in Figure 4.

30 Yet another extraordinary divergence between the two recombinant strains is depicted in Figure 5. This carbon dioxide data comes from the mass spectrometer measurements of the effluent gas. As shown in Figure 5, overexpression of *GDH2* significantly attenu- ates carbon dioxide evolution. Integrated values for CO_2 production from 0 to 30 hours

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are 100.4 and 80.7 mmol/l for the control and *GDH2* strains, respectively, i.e. the *GDH2* strain wastes about 20% less of the carbon source to carbon dioxide production.

Enzymatic assays for NADP-GDH and NAD-GDH were performed on chosen samples
5   and results from two such sets are shown in Figure 6. The assay mixture contained 100 mM sodium phosphate buffer at pH=7.0, 200 μM of either NADPH (NADP-GDH assay) or NADH (NAD-GDH assay), and cell lysate at a final concentration of about 0.5 mg/ml. The reaction was started by addition of 20 mM α-ketoglutarate plus 40 mM NH$_4$Cl, and the NAD(P)H consumption was monitored spectrophotometrically at 340
10   nm. Both strains have notable NADP-GDH activities as expected, although H1803 appears to have a surprisingly higher specific activity for this enzyme (about 40% or so). On the other hand, NAD-GDH activity is essentially close to the assay detection limit for the control strain, whereas, H1803 has a fairly high specific activity for this NAD-enzyme.
15

These results show that the recombinant strain H1803 overexpressing the NAD-glutamate dehydrogenase has significantly enhanced capabilities for ethanol (and xylitol) production, both in terms of higher specific productivities as well as higher product yields on the carbon substrate. The recombinant strain also produces significantly less
20   (undesired) cell mass and very substantially less (undesired) carbon dioxide, thereby not only increasing yields of desired products but also decreasing disposal and pollution loads.

Example 9.   Ethanol Production from Glucose

25

Glucose fermentation to ethanol was conducted in 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of *Saccharomyces cerevisiae*, designated as H1793 and H1791, both of which are derived from *S. cerevisiae* CEN.PK2 (VW-1B) (Boles *et al.* [1996]). Strain H1791 is transformed with a plasmid that expresses the
30   NAD-glutamate dehydrogenase (NAD-GDH) from *S. cerevisiae* (YEplac181+*GDH2*), whereas strain H1793 contains only the cloning vector (YEplac181) and serves as a control strain. Plasmid segregation can be minimized by omitting leucine (LEU) from the growth media.

36

The inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer
flask that contained 50 ml of modified synthetic complete medium without leucine (SC–
LEU) + 20 g/l of glucose. The cells were grown overnight on a rotary shaker at 150
rpm and 30 °C (OD_{600}: 10–15). Cells from the above cultivation were harvested by a

5   10 minute centrifugation at 4,500 rpm and 4°C, washed with 0.1 M PO_4^{2-} buffer
(pH=5.5) and resuspened in the same buffer each to a final volume of 25 ml and
subsequently transferred to the fermentor. The fermentation medium contained (per
liter): yeast–nitrogen–base (without amino acids and without ammonia) 3.4g, uracil
0.044g; tryptophan 0.164g, histidine 0.116g, KNO_3 5.055g, glucose 40g. The fermentor

10 temperature was maintained at 30 °C, the pH was controlled at 5.5 by addition of 2 M
NaOH, and the agitation was constant at 300 rpm. The cultivation was carried out under
anaerobic conditions by flashing the headspace of the fermentor with nitrogen at a
constant flowrate of 0.2 vvm. The offgas was connected via a multi–port valve to a
Balzers quadrupole mass spectrometer (Sweden) for online analysis. Liquid samples

15 were withdrawn from the fermentor at time intervals to measure growth, substrate
consumption, and the formation of extracellular products. Biomass, glucose, ethanol,
glycerol and acetate were measured as in the previous example.

Table 1 summarizes the primary fermentation data for these two fermentations. The
20 NAD–GDH overexpressing strain (H1791) accumulates on the average about 12% less
biomass (0.52 vs. 0.46 g/l). during the 21 hour time period. Specific glucose consump-
tion rates are comparable for the two strains (within 5%). However, the GDH2 strain
has both a higher volumetric (11%) and a higher specific (25%) ethanol production
rate. Yet another extraordinary divergence between the two recombinant strains is

25 depicted in Figure 7. This carbon dioxide data comes from the mass spectrometer
measurements of the effluent gas. As shown in Figure 7, overexpression of NAD–GDH
significantly attenuates carbon dioxide evolution. Integrated values for CO_2 production
from 0 to 21 hours are 93.7 and 70.6 mmol/l for the control and GDH2 strains respect-
ively, i.e. the GDH2 strain wastes about 25% less of the carbon source to carbon

30 dioxide. The specific CO_2 production rate for the GDH2 strain is also attenuated by
about 15%. In addition, the yield of ethanol on glucose (mol/mol) is estimated to be
approximately 19% higher for the GDH2 strain vs. the control strain.

37

These results show that the recombinant strain H1791 overexpressing the NAD–glutamate dehydrogenase (NAD–GDH) has significantly enhanced capabilities for ethanol production from glucose, both in terms of higher specific productivities as well as higher product yields on the carbon substrate. The recombinant strain also produces significantly less (undesired) cell mass and less (undesired) carbon dioxide, thereby not only increasing yields of desired products but also decreasing disposal and pollution loads.

Table 1. Glucose fermentations with *Saccharomyces cerevisiae* recombinant strain overexpressing NAD–GDH (H1791) and control strain (H1793): comparison of growth, glucose consumption, ethanol production and carbon dioxide evolution rates. The last two rows show calculated average fluxes expressed in either volumetric (J_V, mmol/l h) or specific (J_S, mmol/g-cell h) terms. Glucose and ethanol concentrations represent average values from four measurements: two with HPLC and two with enzymatic assays.

	Biomass (g/l)		*Glucose (g/l)*		*Ethanol (g/l)*		CO_2 *(mole %)*	
Time (h)	1793	1791	1793	1791	1793	1791	1793	1791
0	0.315	0.300	39.27	37.57	0.01	0.01	0.000	0.000
6	0.740	0.670	37.68	36.48	0.63	0.72	1.333	0.619
17	0.790	0.760	33.00	32.49	2.39	2.37	1.552	0.823
19	0.820	0.690	32.22	31.04	2.65	2.78	1.735	0.701
21	0.800	0.670	30.98	29.85	2.98	3.30	1.818	0.711
J_V	–	–	–2.19	–2.04	3.08	3.41	4.46	3.36
J_S	–	–	–4.21	–4.43	5.92	7.40	8.58	7.31

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Example 10. An alternative cloning strategy of the open reading frame YKL029C encoding the malic enzyme homologue from *Saccharomyces cerevisiae*

If the restriction enzyme *BglII* is used for the cloning of the malic enzyme homologue
5   between the promoter and terminator of *PGK1* in the yeast expression vector pMA91, a
partial enzyme digestion has to be done, as there is an internal *BglII* site at +227bp of
the malic enzyme coding region. An alternative cloning strategy was as follows:
The PCR fragment was digested with *HindIII* restriction enzyme and treated with
Klenow enzyme to make blunt ends. The expression vector pMA91 (Mellor *et al.*
10 [1983]) was digested with *HindIII* restriction enzyme and the 1.8 kb fragment contain-
ing the *PGK1* promoter and terminator was isolated from an agarose gel. The promoter–
terminator cassette was ligated into the YEplac195 vector (Gietz and Sugino [1988])
which had been linearised at its multicloning site with *HindIII*. The orientation of the
promoter–terminator fragment in the expression vector is *HindIII–PGK1* promoter–
15 *PGK1* terminator–*EcoRI*. This expression vector was digested with *BglII* and treated
with Klenow enzyme to obtain blunt ends for the cloning of the malic enzyme
homologue between the promoter and terminator of *PGK1*.

The ORF YKL029C cloned by PCR was completely sequenced. Two point mutations,
20 created during PCR amplification altering two amino acids in the enzyme were
observed; leucine 341 to valine and glutamine 620 to leucine. These point mutations
were neither in regions of conserved amino acids of malic enzymes (Viljoen *et al.*
[1994]) nor did they significantly alter the apparent K_m values of the PCR–amplified
enzyme as compared to the native enzyme (native enzyme activity measured from the
25 strain H1346; see Table 2 and Fuck *et al.* [1973]) and were thus considered neutral. The
invention can be practised with this gene variant, or with a native gene encoding malic
enzyme or with any other neutral variant.

Table 2. The apparent K_m values for malate, NAD^+ and $NADP^+$ of the native and the PCR-amplified malic enzyme from *S. cerevisiae*

	L-malate K_m (mM)	NAD^+ K_m (mM)	$NADP^+$ K_m (mM)
Native malic enzyme	17.1	0.07	0.06
PCR-amplified malic enzyme	15.4	0.14	0.08

The YEplac195 control vector, and the same vector with the malic enzyme homologue of *S. cerevisiae* under *PGK1* promoter and terminator were transformed into the yeast strain CEN.PK2 (VW-1B, H1346), to obtain strains H2189 (VTT C-99316) and H2193 (VTT C-99317), respectively.

Example 11. Integration of the xylulokinase gene (YGR194C) into the integrant strain H1469

A cassette for the integration of the xylulokinase gene was constructed with *HIS3* flanking regions and the kanamycin resistance gene *KMX2* for selection.

The yeast shuttle vector pRS423 (Christianson *et al.* [1992]) was digested with *DrdI* to obtain a fragment of 1.5 kb containing the *HIS3* gene encoding imidazoleglycerol-P hydratase (Fink [1964]). The fragment was isolated from an agarose gel, blunt ended with T4 polymerase and ligated to the *EcoRV* site of Bluescript SK bacterial cloning vector (Stratagene).

The pFA6-kanMX2 plasmid (Wach *et al.* [1994]) was digested with *PvuII* and *SpeI* to isolate the 1.4 kb kanamycin resistance module. The *KMX2* fragment was isolated from an agarose gel and blunt ended with Klenow enzyme. The Bluescript SK carrying the *HIS3 DrdI* fragment was digested with *NheI* and *BclI* within the *HIS3* gene (removing 50 bp from its coding region), blunt ended with Klenow enzyme and treated with shrimp alkaline phosphatase, prior to ligation with the isolated *KMX2* fragment.

40

The xylulokinase gene under the modified *ADH1* promoter and *ADH1* terminator in
YEplac195 (see Example 2.) was isolated after a *BamHI* digestion from an agarose gel.
The Bluescript SK carrying the *HIS3 DrdI* fragment with the *KMX2* module was
digested with *BglII* within the *HIS3* gene (two adjacent *BglII* sites removing 60 bp from
5   its coding region) and was treated with shrimp alkaline phosphatase, prior to ligation
with the isolated xylulokinase fragment with *ADH1* promoter and terminator.

The final integration cassette thus constructed contained 900 bp of *HIS3* sequence, the
xylulokinase gene (*ADH1* terminator, the XK gene, modified *ADH1* promoter) 50 bp
10   *HIS3* coding region, the *KMX2* cassette (promoter, the gene, terminator) and 550 bp of
HIS3 sequence. This construction was separated from Bluescript SK by *BstBI* and *BssHI*
digestion, leaving about 400 bp of *HIS3* sequence at either side for targetted integration.

One μg of the integration cassette isolated from an agarose gel was used to transform
15   the H1469 strain, to obtain the strain H2217 (VTT C-99318). The screening for
integrant transformants was on YPD plates with 200 μg/ml of G418. The correct
integration of the XK cassette to the *HIS3* locus was verified by Southern hybridisation,
by PCR, and by enhanced growth on xylulose as compared to the wild type.

20   **Example 12. Transformation of the integrant strains H1469 and H2217 with the
gene encoding malic enzyme on a multicopy expression vector**

The above described plasmid YEplac195 carrying the gene *MAE1* encoding the malic
enzyme under the promoter and terminator of *PGK1* (see example 6 or 10), and the
25   control plasmid YEplac195 were transformed into the integrant strains H1469 and
H2217. The plasmids were selected for by omitting uracil from the growth medium.
Plasmid rescue from the yeast transformants verified the integrity of the control and
expression plasmids. The strains obtained were named as follows: H1469 with the
control plasmid as H2191 (VTT C-99319), H1469 with the malic enzyme plasmid as
30   H2195 (VTT C-99320), H2217 with the control plasmid as H2221 (VTT C-99321) and
H2217 with the malic enzyme plasmid as H2222 (VTT C-99322).

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Example 13. Effect of Malic Enzyme: Ethan l Production from Gluc se

Glucose fermentation to ethanol was conducted in 1.8 liter Chemap CMF Fermentor
(Switzerland) by genetically engineered strains of *Saccharomyces cerevisiae* derived
from *S. cerevisiae* CEN.PK2/ VW-1b (Boles *et al.* [1996]) designated below as H1346.
More specifically, the two strains used is this example are as follows: (i) H2193: H1346
transformed with a plasmid that expresses the *S. cerevisiae* malic enzyme (MAE1, ORF
YKL029c), or, (ii) H2189: H1346 transformed with the cloning vector (YEplac195) and
serves as a control strain. Omitting uracil (URA) from the growth media minimizes
plasmid segregation.

The inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer
flask that contained 50 ml of synthetic complete medium without uracil (SC-URA) +
20 g/l of glucose. The cells were grown overnight on a rotary shaker at 150 rpm and
30 °C (OD_{600}: 10-15). Cells from the above cultivation were harvested by a 10 minute
centrifugation at 4,500 rpm and 4°C, washed with 0.1 M phosphate buffer (pH=5.5) and
resuspended in the same buffer each to a final volume of 25 ml and subsequently
transferred to the fermentor. The fermentation medium contained (per liter): yeast-
nitrogen-base (without amino acids), amino acid supplements, glucose 30g. The
fermentor temperature was maintained at 30 °C, the pH was controlled at 5.5 by
addition of 2 M NaOH, and the agitation was constant at 300 rpm. The cultivation was
carried out under anaerobic conditions by sparging the broth with nitrogen at a constant
flow rate of 0.1 vvm. Liquid samples were withdrawn from the fermentor at time
intervals to measure growth, substrate consumption, and the formation of extracellular
products. Biomass, glucose, ethanol, glycerol and acetate were measured as in the
previous example.

Table 3 summarizes the fermentation results for these two fermentations. The MAE1
overexpressing strain (H2193) utilizes approximately 20% less carbon for biomass
synthesis (3.27 vs. 2.60 C-mol/g-cell h), resulting in final biomass densities that are
significantly lower compared with the control (1.33 vs. 2.20 g/l). The specific glucose
consumption rate is ca. 20% higher for the MAE1 strain. More importantly, the MAE1
has higher specific ethanol production rate of about 25% (18.85 vs. 23.64 C-mmol/g-

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cell h). Higher specific ethanol production rates are advantageous in processes that are physically constrained by the amount of biocatalyst that can be utilized, e.g. cell–immobilized fermentation systems. Furthermore, the ethanol yield on glucose for the MAE1 overexpressing strain is about 4% higher compared with the control (0.478 vs. 0.499 C–mol/C–mol).

These results disclose that the recombinant *S. cerevisiae* strain H2193 overexpressing the endogenous malic enzyme (MAE1) has significantly enhanced capabilities for ethanol production from glucose as the carbon substrate. The recombinant strain also produces significantly less (undesired) cell mass, thereby not only increasing yields of desired products but also decreasing disposal loads.

Table 3. Glucose fermentations with *Saccharomyces cerevisiae* recombinant strain expressing *MAE1* (H2193) and control strain (H2189): average fluxes expressed in either volumetric (J_v, C–mmol/l h) or specific (J_s, C–mmol/g–cell h) terms (time interval: 3.3 to 29 hours). Glucose and ethanol concentrations represent average values from four measurements: two with HPLC and two with enzymatic assays.

	Biomass (g/l)		Glucose (g/l)		Ethanol (g/l)	
	H2193	H2186	H2193	H2186	H2193	H2186
J_v (C–mmol/l h)	1.68	3.01	30.61	36.32	15.27	17.35
J_s (C–mmol/g–cell h)	2.60	3.27	47.37	39.47	23.64	18.85

The previously described experiment was repeated with the following modifications: (1) The bioreactor inoculum size was increased from ca. 0.1 g/l to ca. 2 g/l, and, (2) In addition to using a large inoculum (ca. 2 g/l), subsequent to glucose exhaustion (24 hours) a concentrated solution of glucose was added to the fermentor to give a final glucose concentration of about 45 g/l and the fermentation was allowed to proceed for an additional 24 hours (repeated batch). The primary results were as follows for the two cases: (1) Overexpression of the malic enzyme results in higher specific rates of ethanol production (+9%) and glucose consumption (+20%) with less glucose being

diverted to biomass for (−16%); (2) Overexpression of the malic enzyme enhanced the specific ethanol production rates by 6 and 8 % for the first and second phases respectively, while corresponding glucose uptake rates are also higher by 16 and 17 % in the two phases. The initial biomass concentration of 2 g/l for both strains increased to 5.73

5   and 6.36 g/l for the strain overproducing malic enzyme and the control respectively during the first phase (i.e. 10% less for MAE1). Corresponding values for the second phase were 7.66 vs. 8.15 g/l (i.e. 6% less for MAE1).

To summarize, these three experiments disclose that the recombinant S. cerevisiae strain

10   H2193 overexpressing the native malic enzyme (MAE1) has significantly enhanced capabilities for ethanol production from glucose as the carbon substrate (specific rates increased by up to 25%, yields increased by 4%). The recombinant strain also produces significantly less (undesired) cell mass, thereby not only increasing yields of desired products but also decreasing disposal loads.

15

Example 14. Effect of Malic Enzyme: Ethanol Production from Xylose

Xylose fermentation to ethanol was conducted in a 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of Saccharomyces cerevisiae derived

20   from S. cerevisiae CEN.PK2/ VW-1b (Boles et al. [1996]) designated below as H1346. More specifically, the four strains used is this example are as follows: (i) **H2195**: H1346 carrying chromosomal integrations of genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) (strain H1469) transformed with a plasmid that expresses the S. cerevisiae malic enzyme (MAE1, ORF YKL029c) (ii) **H2191**: H1469 transformed

25   with the cloning vector (YEplac195) and serving as a control for strain H2195. (iii) **H2222**: H1346 carrying chromosomal integrations of genes encoding xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) (strain H2217) transformed with a plasmid that expresses the S. cerevisiae malic enzyme (MAE1, ORF YKL029c) (iv) **H2221**: H2217 transformed with the cloning vector (YEplac195) and serving as a

30   control for strain H2222. Omitting uracil (URA) from the growth media minimizes plasmid segregation.

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The inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer flask that contained 50 ml of synthetic complete medium without uracil (SC–URA) + 0.1 M phosphate + 20 g/l of glucose. Cells were grown overnight on a rotary shaker at 150 rpm, 30°C and then the whole broth was transferred to a 2 L flask that contained

5   500 ml SC–URA + 0.1 M phosphate + 50 g/l glucose. The culture was again grown overnight as above and the cells were then harvested by a 10 minute centrifugation at 4,500 rpm and 4°C. The cells were then washed with 0.1 M phosphate buffer (pH=5.5) and resuspended in the same buffer each to a final volume of 100 ml and subsequently transferred to the fermentor.

10

The fermentation medium contained (per liter): yeast–nitrogen–base (without amino acids), amino acid supplements, xylose 50g. The fermentor temperature was maintained at 30 °C, the pH was controlled at 5.5 by addition of 2 M NaOH, and the agitation was constant at 300 rpm. The cultivation was carried out under anaerobic conditions by

15   sparging the broth with nitrogen at a constant flowrate of 0.1 vvm. Liquid samples were withdrawn from the fermentor at time intervals to measure growth, substrate consumption, and the formation of extracellular products. Biomass, glucose, ethanol, glycerol and acetate were measured as in the previous example.

20   a) Anaerobic xylose fermentations with strains H2195 and H2191

Figure 8 shows time profiles for the biomass and turbidity for the MAE1 overexpressing strain (H2195) and the control strain (H2191). Note that *Saccharomyces cerevisiae* expressing genes encoding XR and XDH in general can utilize xylose, however, they

25   are unable to grow on xylose anaerobically. As illustrated by Figure 8, overexpression of the native malic enzyme has indeed a positive effect on cell viability under these conditions. Strain H2195 portrays an initial growth on xylose even under anaerobic conditions which is followed by a decline in biomass to a level after 32 hours that corresponds to that of the inoculum. On the contrary, the biomass of the control

30   declines monotonically during this same time period from an initial value of 2.11 down to 1.47 g/l, i.e. a drop of biomass of ca. 30%.

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Figure 9 summarizes the overall specific metabolic rates (C-mmol/g-cell h) for these
two batch fermentations. Evidently, in addition to preventing cell degradation, over-
expression of the malic enzyme has also a positive effect on xylose utilization and on
the production of both ethanol and xylitol. Xylose utilization is enhanced by more than

5   55%, and the productivities of ethanol and xylitol are increased by about 20% and
25%, respectively.

These results disclose that the recombinant *S. cerevisiae* strain H2195 overexpressing
the native malic enzyme (MAE1) has significantly enhanced capabilities for xylose

10   utilization as well as for ethanol and xylitol production from xylose. The recombinant
strain can also sustain its viability for prolonged time periods.

b) Anaerobic xylose fermentations with strains H2222 and H2221

15   This experiment was carried out as described in part (a) except that strains H2195 and
H2191 were substituted with strains H2222 and H2221 that in addition to the genes
encoding XR and XDH also carry a chromosomal integration of a gene encoding the
native XK. Strain H2222 overexpresses the native malic enzyme.

20   It is evident from Figure 10 that overexpression of the malic enzyme has a significant
enhancing effect on ethanol production from xylose, and also that this can be sustained
for several days in a row.

As indicated in Figure 11, the enhancement of specific ethanol production rate is close

25   to 30% compared with the control, and the specific xylose utilization is up by about 5
%. Furthermore, as indicated by Figure 12, the control strain accumulates a signifi-
cantly higher amount of biomass over this 144-hr time period (2.03 vs. 1.47 g/l, i.e. ca
+40%). This behavior appears to be different as compared with strains H2195 and
H2191 described in part (a) above. A plausible cause for this difference can lie with XK

30   which can play a significant role in xylose utilization (note that H2222 and H2221 carry
chromosomal integrations of native XK). However, the reduction in biomass observed in
part (b) is in good agreement with that observed in example 13 that deals with the
effect of MAE1 on glucose conversion to ethanol. In addition, the molar yield of

ethanol on xylose was about 25% higher for the strain that overexpresses the malic enzyme: 0.114 vs. 0.091 C-mol ethanol per C-mol xylose.

5   In summary, these experiments disclose that the recombinant *S. cerevisiae* strains H2195 and H2222 overexpressing the native malic enzyme (MAE1) in genetic backgrounds without and with XK, respectively, have significantly enhanced capabilities for xylose utilization and ethanol production from xylose as the carbon substrate (up to 30%). In addition, strain H2222 produces less (undesired) acetate (~30%). In the background lacking XK only the microorganism transformed according to the invention (H2195) is able to survive under the process conditions. On the other hand, in the background with XK, where the non-transformed microorganism can survive, the microorganism transformed according to the invention (H2222) produces significantly less (undesired) cell mass (up to 40%), thereby not only increasing yields of desired products but also decreasing disposal loads.

Example 15: Construction of an integration vector for expression of the gene encoding malic enzyme from *S. cerevisiae* in *Schizosaccharomyces pombe*

The YEplac195 vector with the malic enzyme including *PGK* promoter and terminator from example 10 was digested with *HindIII* and the 3.7 kbp fragment isolated from an agarose gel. This fragment was treated with Klenow enzyme to make blunt ends. This fragment was then ligated to the *SmaI* site of the vector pJK210 (ATCC86957). The vector was digested with *AvrII* for integration by homologous recombination in the *URA4* gene.

Example 16: Construction of a multicopy vector for expression of the genes encoding xylose reductase and xylitol dehydrogenase of *P. stipitis* in *Schizosaccharomyces pombe*

The *SacI* and *NsiI* fragment from Example 1, containing the gene for xylose reductase under *PGK* promoter and terminator and the gene for xylitol dehydrogenase under a modified *ADH1* promoter and terminator was isolated from an agarose gel. It was then

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ligated to the vector pSP1 (ATCC77497), which contains the *LEU2* gene, which was linearized by digesting with PstI and *SacI.*

Example 17: Transformation of *Schizosaccharomyces pombe*

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The linearised vector from example 15 was transformed by electroporation (http://www.bio.uva.nl/pombe/handbook) to the *S. pombe* strain ATCC201400. A control strain was made in a similar way by integrating the empty vector pJK210. The transformants were selected for URA auxotrophy. The multicopy vector from example 16 was

10   transformed in the same way. Transformants were selected for additional LEU auxotrophy. The resulting strain is called H2369 (VTT C-99323) and the control strain without malic enzyme activity H2370 (VTT C-99324).

Example 18. Effect of Malic Enzyme: Ethanol Production from Xylose with

15   *Schizosaccharomyces pombe*

Xylose fermentation to ethanol was conducted in a 1.8 liter Chemap CMF Fermentor (Switzerland) by genetically engineered strains of *Schizosaccharomyces pombe* derived from *S. pombe* strain H2153 (ATCC 201400). More specifically, the two strains used in

20   this example are as follows: (i) **H2369**: H2153 carrying a chromosomal integration of the *S. cerevisiae* gene encoding malic enzyme and transformed with a multi-copy plasmid that expresses the genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) of *Pichia stipitis*. (ii) **H2370**: H2153 carrying a chromosomal integration of the cloning vector pJK210 and transformed with a multi-copy plasmid

25   that expresses genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) of *Pichia stipitis*. Omitting uracil (URA) and leucine (LEU) from the growth media minimizes plasmid segregation.

The inoculum was prepared by transferring a single colony into a 250 ml Erlenmeyer

30   flask that contained 20 ml of Edinburgh Minimal Medium (http://www.bio.uva.nl/ pombe/handbook/section1/section1-8.html) with 225 mg/l adenine, histidine and lysine hydrochloride added (EMM2+ADE+HIS+LYS) + 20 g/l of glucose. Cells were grown about 50 hours on a rotary shaker at 200 rpm, 30°C and then the whole broth was

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transferred to a another 250 ml Erlenmeyer flask that contained 50 ml of the same medium. Cells were grown about 24 hours on a rotary shaker at 200 rpm, 30°C and then the whole broth was transferred to a 2 L flask that contained 700 ml EMM2 + ADE+HIS+LYS + 50 g/l glucose. The culture was grown for about 40 hours as above

5   and the cells were then harvested by a 10 minute centrifugation at 4,000 rpm and 4°C. The cells were then washed with 0.1 M phosphate buffer (pH=5.5) and resuspended in the same buffer each to a final volume of 100 ml, OD 600 of the both strains were adjusted to same value with buffer and subsequently transferred to the fermentor. The fermentation medium contained EMM2 + ADE (225 mg/L) + HIS (450 mg/L) + LYS

10   (450 mg/L) + 50 g/L xylose.

The fermentor temperature was maintained at 30 °C, the pH was controlled at 5.5 by addition of 1 M KOH, and the agitation was constant at 300 rpm. The cultivation was carried out under microaerobic conditions by sparging the broth at a constant flowrate

15   of 0.5 SLPM with mixture of nitrogen and air (7:1), the fraction of oxygen in the inlet thus being 2.5 %. Liquid samples were withdrawn from the fermentor at time intervals to measure growth, substrate consumption, and the formation of extracellular products. Biomass was measured according to OD_{600} and measuring the dry weight of samples. Xylose, ethanol, xylitol, glycerol and acetate were measured with HPLC. Ethanol was

20   also measured by enzymatic assay with Cobas – Mira.

Figure 13 shows time profiles for the biomass and turbidity for the strain H2369 and the control strain H2370. As illustrated by Figure 13, expression of the malic enzyme has indeed a positive effect on cell viability under these conditions. Decline in biomass

25   is from 2.6 to 1.9 g/l with H2369 and from 2.43 to 1.2 g/l with the control strain H2370. Starting at very similar biomass levels, the control decreases by more than 50 % and the strain transformed according to the invention by less than 30 %. Thus, this is another example disclosing that under conditions where control strains have a poor ability to survive and maintain their biomass and metabolic capacity, strains transformed

30   according to the invention have an improved ability to maintain their biomass and consequently their metabolic capacity. The logarithmic averages of the biomasses during fermentation are 2.23 g/l for H2369 and 1.74 g/l for H2370.

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Figure 14 shows the volumetric ethanol production rates with the strain H2369 and the control strain H2370. As illustrated by Figure 14 the volumetric ethanol production with H2369 is significantly higher compared to the control strain H2370 (+62 %). The specific ethanol production rate is higher by 26 % (0.386 vs 0.305 C-mmol/g-cell ·h).

5   Evidently, in addition to preventing cell degradation, expression of the malic enzyme has also a positive effect on ethanol production. Volumetric xylose utilization is enhanced by 47 % (9.7 g/l vs 6.6 g/l). Also the specific rate is enhanced (by about 15 %; 1.45 vs 1.26 C-mmol/g-cell ·h, results not shown here), showing that the metabolic capacity of the extra biomass maintained by the transformed strain was even greater

10   than that of the control strain.

These results disclose that the recombinant *S. pombe* strain H2369 expressing the *S. cerevisiae* malic enzyme has significantly enhanced capabilities for xylose utilization as well as for ethanol production from xylose. The recombinant strain can also sustain its

15   viability for prolonged time periods. In addition, strain H2369 produces less (undesired) acetate (−43%). These results are comparable with fermentations by *S. cerevisiae* over-expressing native malic enzyme. With *S. cerevisiae* xylose utilization was enhanced by 55 % and ethanol production by 20 %.

20   **Example 19: Cloning the gene for NAD-linked glutamate dehydrogenase from *Peptostreptococcus asaccharolyticus***

Peptostreptococcus asaccharolyticus (ATCC 14963) was grown anacrobically and genomic DNA isolated. The gene encoding NAD-linked glutamate dehydrogenase was

25   then cloned according to the sequence published by Snedcor *et al.* (1991) by PCR, using the following oligonucleotides: 5'-GAG GAT CCA TAG GAG CGC ATG TTG GAC (SEQ ID NO: 13) (SEQ ID NO: 14) C-3' and 5'-CAG GAT CCT CTG TTA GGG ATT TAC TCC-3'. DyNAzyme EXT polymerase (Finnzymes) was used and the resulting 2.4 kbp PCR product was ligated to the pCR 2.1 TOPO vector (Invitrogen) and transformed to TOP10F' *E. coli* cells

30   (Invitrogen) according to the manufacturer's instructions.

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Example 20: Construction of an *E. coli* – *Corynebacterium glutamicum* shuttle vector containing the gene encoding the NAD–linked glutamate dehydrogenase from *Peptostreptococcus asaccharolyticus*

5   The plasmid pAJ655, an *E. coli* – *Corynebacterium glutamicum* shuttle vector, was isolated from the *Corynebacterium glutamicum* strain ATCC 39135 and digested with *BamHI*. The vector was purified on an agarose gel and the ends dephosphorylated with shrimp alkaline phosphatase.

10   The pCR 2.1 TOPO vector with the gene coding for the NAD–linked glutamate dehydrogenase from *Peptostreptococcus asaccharolyticus* from Example 19 was digested with BamHI and the 2.4 kbp fragment isolated from an agarose gel. The 2.4 kbp fragment was then ligated to the linearised shuttle vector and transformed to *E. coli* DH5α cells.

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Example 21: Transformation of the shuttle vector to a strain of *Corynebacterium glutamicum*

The strains of *Corynebacterium glutamicum* ATCC 21799 (E–991193) and ATCC

20   21253 (E–991192) were transformed with the shuttle vector obtained in Example 20 by electroporation (Follettie [1989]). These strains were chosen because they have been developed to overproduce lysine.

The plasmid was recovered from the transformed strains, digested with *BamHI* and the

25   digestion products separated on an agarose gel as shown in Figure 15. In lane 1 is the digested vector from a transformant of ATCC 21253 and in lane 3 is the digested vector from a transformant of ATCC 21799 which is called VTT E–992103. The figure shows the 10 kbp vector and the 2.4 kbp insert. A second isolate of transformed ATCC 21799 was named VTT E–992104, and the plasmid recovered from this isolate behaved in the

30   same way as shown for VTT E–992103 in Fig. 15.

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Example 22: Measuring NAD–linked glutamate dehydrogenase activity in cell extracts of *Corynebacterium glutamicum*

Cell extracts of *Corynebacterium glutamicum* cells were prepared by vortexing 500 mg
wet weight of cells, 500 μl 100 mM sodium phosphate buffer pH 7.0 and 500 μl glass
beads for 20 minutes at 4 °C in 1.5 ml Eppendorf tubes. The tubes were centrifuged at 4
°C and 13000 rpm in a table top centrifuge and the supernatant analysed. The buffer for
the activity assay contained 100 mM sodium phosphate pH 7.0, 20 mM ammonium
chloride and 200 μM NADH. The reaction was started by adding α–ketoglutarate to a
final concentration of 2 mM. The activity was calculated from the change in NADH
absorbance at 340nm. The enzyme assays were done at 30 °C. The amount of extracted
protein was measured with the BioRad protein assay using IgG as a standard. Enzyme
activities between 0.1–0.2 nkat/mg were found for a transformant of the ATCC 21253
strain and 0.1–0.2 nkat/mg for transformants of the strain ATCC21799 which are called
VTT E–991203 and VTT E–991204.

Example 23: Lysine production by transformed *Corynebacterium glutamicum*

Fermentation in shake flasks: The strains VTT E–991203 and VTT E–991204 were
analysed in shake flask fermentations as described by Kiss (1991), except that the
antibiotic used was 10 mg/l chloramphenicol instead of kanamycin. As a control the
parent strain ATCC 21799 was grown in the absence of an antibiotic. 1 ml of a
preculture, grown on LB, was diluted into 50 ml CGM1 medium (Kiss [1991]) and
grown at 30 °C in baffled 250 ml Erlenmeyer flasks on a shaker at 250 rpm. The amino
acids in the supernatants were analysed by HPLC and glucose with an enzyme assay.
Results are shown in Table 4.

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Table 4. Production of lysine by a contr 1 strain (ATCC21799) and two transformants according to the invention of *Corynebacterium glutamicum*.

	Biomass (g dry wt./l)	Glucose utilized (g/l)	Threonine utilized (mg/l)	Lysine produced (g/g biomass)	Lysine produced (g/g glucose utilized)
VTT E-991203	0.855	3.3 (16.5%)	73.6 (49%)	0.408	0.105
VTT E-991204	1.236	3.5 (17.5%)	94.8 (63%)	0.426	0.150
ATCC 21799	4.93	13.7 (68.5%)	144.7 (96%)	0.484	0.173

The two transformed strains VTT E-991203 and VTT E-991204 grew more slowly than the control because (unlike the control) they contain shuttle vectors and were cultivated in the presence of chloramphenicol. When lysine production was measured, the transformants were still in phase I of the fermentation, i.e. threonine was still present, whereas the control was close to or in stationary phase and had consumed nearly all the threonine. In *Corynebacteria* overproduction of L-lysine starts only in phase II, after the threonine is utilized (Vallino [1991]). Remarkably, however, the transformants already produced lysine during phase I, and the amounts of lysine produced per gram biomass were only 16 % and 12 % less than those produced by the control in the normal lysine over-producing stage (phase II). Furthermore, the yields of lysine on glucose utilised obtained with the transformants markedly increased as the transformants grew through phase I (from 0.105 g/g when 49 % of threonine was consumed to 0.15 g/g when 63 % of threonine was consumed). For conventional lysine-overproducing strains of *Corynebacterium glutamicum* the lysine yields on glucose utilised expected at this stage of the fermentation are close to zero (Vallino [1991]). It is evident that when the transformants emerge from phase I into the normal lysine overproduction stage (when growth is complete, and glucose is no longer diverted to biomass production) the rates and yields of lysine production will exceed those of the control. It is clear to those skilled in the art that the slow growth of the transformants can be accelerated by integrating the gene for NAD-linked glutamate dehydrogenase

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into the transformants' genomes, thereby avoiding the handicap of supporting a plasmid and eliminating the need for chloramphenicol in the growth medium.

5 This example discloses that transformation of a lysine–overproducing strain of *Coryne–bacterium glutamicum* with a gene for NAD–linked glutamate dehydrogenase according to the invention enhances the production of lysine by the transformant at least during early growth stages. It is expected that substantial enhancement of lysine yields on glucose will be obtained when the fermentations with transformants are continued into phase II.

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Example 24. Coexpression of the genes from *Alcaligenes eutrophus* encoding polyhydroxybutyrate (PHB) reductase and PHB synthase with the gene encoding NAD–dependent glutamate dehydrogenase or the gene encoding malic enzyme of *S. cerevisiae*

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Plasmid pRS303 (Sikorski and Hieter [1989]) was digested with *SacI* and *XbaI* and isolated from a 0.7% agarose gel. The 4.8 kbp *GDH2* fragment (containing the gene with its own promoter) on a *SacI / XbaI* cassette (Boles *et al.* [1993]) was ligated into the pRS303 vector creating the plasmid pGDH2–303. The 2 micron origin of replication

20 was cut from the plasmid pLGSD5 (Guarente *et al.* [1982]) with *EcoRI* and the ends rendered blunt with the Klenow Large Fragment Polymerase. Plasmid pRS303 was digested with *XhoI* and the ends rendered blunt with the Klenow Large Fragment Polymerase. The origin of replication was then ligated into pRS303 creating plasmid pTL92.

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Plasmid pGDH2–303 was digested with *SmaI* and a doublet band of ¯4.8 kbp contain-ing both the *GDH2* fragment (4.8 kbp) and the backbone vector (4.5 kbp) was isolated from an agarose gel. The recovered fragments were then digested with *KpnI*. This digestion cleaves the backbone vector (pRS303) into two fragments thus allowing the

30 isolation of the 4.8 kbp band containing the *GDH2* fragment. Plasmid pTL92 was linearized with *SmaI* and isolated from an agarose gel. The linearized vector was dephosphorylated with CIAP (calf intestinal alkaline phosphatase) at 37°C for one hour

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and then purified on an agarose gel. The blunt *GDH2* fragment and blunt vector pTL92
were ligated creating p2–GDH2.

The plasmid p2–GDH2 can be transformed into *S. cerevisiae* strain D603 (*MATa/MATα,*
5   *ura3–52, lys2–801, met, his3, ade2–101, reg1–501*; Srienc *et al.* [1986]) expressing
the heterologous genes from *Alcaligenes eutrophus* encoding PHB reductase and PHB
synthase on a multicopy plasmid p2DPT with a bidirectional, inducible galactose
promoter (Carlson [1999]). Shake flask and bioreactor cultivations with appropriate
controls can be performed in enriched SD media (DaSilva [1988]) containing 10 g/l of
10 each glucose and galactose. The PHB content of control cells and cells transformed
according to the invention are determined by GC analysis of dichloroethane extracts of
dried cell material subjected to propanolysis (Riis and Mai [1988], Leaf *et al.* [1996]).
Sugar consumption and gas exchanges are measured by standard methods. In this way
the advantages expected from the invention, such as increased yield of PHB on glucose,
15 increased productivity, decreased CO_2 production and decreased oxygen requirement can
be demonstrated.

It is clear that the 3.8 kbp fragment with the *MAE1* gene between the promoter and
terminator of *PGK1* (see example 10) can be cloned and expressed in the yeast D603
20 with a similar strategy as used for the *GDH2* gene.

Similar strategies can be used to introduce genes for dehydrogenases according to the
invention into other microorganisms producing PHB and other PHAs, including bacteria
already used in the industrial production of PHAs, such as *Alcaligenes eutrophus.*
25 Advantageous enzymes include the NAD–linked glutamate dehydrogenase from
Peptostreptococcus asaccharolyticus used in Example 21 to transform another bacter-
ium, *Corynebacterium glutamicum.*

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Deposited micr organisms

The following microorganisms were deposited under the rules of the Budapest Treaty at the DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D–38124 Braunschweig, Germany

Microorganism	Accession number	Date of deposit
Saccharomyces cerevisiae		
H1791 (VTT C–98298)	DSM 12213	4 June 1998
H1795 (VTT C–98300)	DSM 12214	4 June 1998
H1803 (VTT C–98302)	DSM 12215	4 June 1998
H2193 (VTT C–99317)	DSM 12722	5 March 1999
H2195 (VTT C–99320)	DSM 12723	5 March 1999
H2222 (VTT C–99322)	DSM 12724	5 march 1999
Schizosaccharomyces pombe		
H2369 (VTT C–99323)	DSM 12725	5 March 1999
H2370 (VTT C–99324)	DSM 12726	5 march 1999
Corynebacterium		
VTT E–991203	DSM	11 March 1999
VTT E–991204	DSM	11 March 1999

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SEQUENCE LISTING

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Thr Ala Leu Leu Asn Ser Pro Ile Phe Asn Lys Gly Ser Ala Phe Pro
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Glu Gln Thr Leu Asp Asn Gln Val Lys Arg Ala Tyr Gln Gln Tyr Gln
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Ser Arg Gly Asp Asp Trp Pro Arg Thr Val Pro Asp
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